THE BIOSYNTHESIS OF A BIOTIN COMPOUND CONTAINING ^{75}Se

BY

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INTRODUCTION

Knowledge of the biological activity of selenium became important during the 1930's when it was demonstrated that various diseases of cattle were caused by ingestion of vegetation containing abnormally high levels of selenium (Byers, 1935). As early as 1935 (Byers, 1935) it had been noticed that an abundance of sulfur in the appropriate form would reduce selenium absorption by plants. This observation coupled with the fact that selenium and sulfur have the same electronic configuration in their outer shell led to speculation that selenium might follow the sulfur metabolic pathways, and that it might exert its toxic effects by inhibiting certain reactions in these pathways or by causing the formation of non-functional selenium analogs of essential sulfur compounds. In 1949, Klug *et al.* reported that selenomethionine and selenocystine were toxic to rats, lending support to the toxic sulfur analog theory.

This thesis was undertaken in order to gain a more complete understanding of the penetration of the sulfur metabolic pathways by selenium. Specifically, an attempt was made to determine if selenium could replace sulfur in biotin synthesized by *Phycomyces blakesleeanus*. Although selenium has been found to replace sulfur in many biologically active compounds, it has never been demonstrated in a

naturally synthesized coenzyme or cyclic structure such as that found in biotin.

Selenium in Sulfur Metabolic Pathways

A number of different approaches have been used to study the metabolism of selenium. Because of the relative ease with which they may be grown and analyzed biochemically, microorganisms have often been used in such studies.

One of the first documented instances of selenium following a sulfur pathway was that of the competitive antagonism which exists between sulfate, an essential nutrient, and selenate, its selenium analog. Fels and Cheldelin (1949) reported that half maximum growth of Saccharomyces cerevisiae was obtained with a relatively constant ratio of selenate to sulfate regardless of the actual concentration of either. In 1954, Shrift published a more conclusive report of this competitive antagonism. He showed that the growth rate of Chlorella vulgaris remained the same with a constant ratio of selenate to sulfate, and that, the amount of selenium incorporated into the cells was dependent on the sulfate/selenate ratio in the growth medium and independent of the actual selenium concentration. From this evidence Shrift hypothosized that the initial anatagonism between sulfate and selenate was competition for transportation sites on the cell membrane. A similar conclusion was reached by Weissman and Trelease

(1955) in that they found the growth of Aspergillus niger to be dependent on the sulfate/selenate ratio rather than on the concentration of selenate in the growth medium. More recently, Tweedie and Segal (1970) have presented evidence that SO_4^{-2} and SeO_4^{-2} enter the cells of some species of Penicillium and Aspergillus by the action of a single permease. Therefore, it appears that selenate uses the sulfate pathway in gaining entrance into the cells of S. cerevisiae, Penicillium sp., Aspergillus sp., C. vulgaris and A. niger, and probably other organisms.

A study of the utilization of selenate by bacteria was published in 1962 by Shrift and Kelly. They reported the adaptation of Escherichia coli to grow in a medium containing equal concentrations of potassium selenate and potassium sulfate. Work on the utilization of selenate continued and in 1967 Huber et al. reported that their substrain of E. coli K-12, adapted to grow in 0.01M sodium selenate, could have only 30-40% of its required sulfur replaced by selenium. They reported that selenate grown cells had a longer than normal lag phase and that growth was essentially linear rather than exponential as was the growth of Shrift and Kelly's (1962) culture. Huber et al. also reported the presence of selenomethionine in their selenate grown E. coli.

Selenomethionine was earlier identified in E. coli grown in radioactive selenite by Tuve and Williams (1961). Thus, both selenite and selenate appear to participate in

selenomethionine synthesis. However, some of the selenite supplied to many microorganisms is reduced to elemental selenium or to dimethylselenide and is thus effectly removed from the organism. Ahluwalia et al. (1968) report that selenite can be reduced to elemental selenium by a constitutive enzyme of E. coli. They also noted that the rate of reduction was unrelated to the rate of growth. In fact, dying cultures with decreasing amounts of nitrogen may continue to accumulate selenium by reducing selenite.

Earlier, Weiss et al. (1965) had reported a correlation between the ability of certain bacteria to reduce selenite and their tolerance of that compound. Apparently, selenite reduction led to non-assimilation of selenium and thus allowed some bacteria to withstand much higher concentrations of selenite than others with slower reduction rates.

Certain fungi are reported to reduce selenite to dimethyl selenide (Barker and Fleming, 1974; Challenger, 1951). This reduction may also serve as a method of rendering otherwise toxic selenite harmless. Thus, it appears that certain organisms rid themselves of toxic selenite by reducing it to less toxic forms.

Another study on the effects of a selenium analog was that of Cowie and Cohen (1957), who demonstrated that the methionine requirement of a methionine requiring strain of Escherichia coli could be satisfied by selenomethionine. This strain of E. coli (ML 304d) grown with selenomethionine produced an active β-galactosidase, although the activity of

the enzyme per unit of dry weight of cells was less than with an enzyme synthesized in the presence of methionine. On the other hand their results indicated that selenium could not replace the entire sulfur requirement of this strain of E. coli.

Further work on the utilization of selenomethionine was published in 1971 by Coch and Greene. They showed that selenomethionine was relatively nontoxic to E. coli 26 especially when cysteine was supplied. Selenomethionine at a concentration of 1×10^{-4} M caused a 95% inhibition of the endogenous production of methionine but did not alter the general rate of protein synthesis. They also found that the induction of β-galactosidase was decreased in the presence of selenomethionine, but that the enzyme remained totally active with 70-75% of its methionine replaced by selenomethionine.

Yet another approach to the question of selenium in the sulfur pathways has been to look for selenium analogs of sulfur metabolites in organisms supplied with a source of selenium. The presence of selenium in the tRNA of E. coli grown with selenite has been demonstrated (Saelinger et al., 1972). Selenium containing bases were isolated and one has been identified as 4-selenouridine (Hoffman and McConnell, 1974). The biological activity of these bases is presently unknown. Though it is generally assumed that selenium is replacing sulfur in these bases, this has not been proven and the possibility remains that selenium may

naturally occur in tRNA.

Shrift (1967) has reported preliminary evidence for the synthesis of selenothiamine by E. coli trained to grow in a miminal medium containing $1x10^{-2}$M potassium selenate, without added sulfur. Sulfur, however was found as a contaminant in the selenate (unpublished data). When this E. coli was grown with ^{75}Se-selenate, chromatography of the cellular extract on a Decalso column, specific for thiamine, produced a peak of radioactivity which was coincident with a peak of thiamine. When this peak was chromatographed on paper, three spots, one with an Rf similar to thiamine appeared. But when rechromatographed in the same solvent system, the "thiamine" spot decomposed into three spots, one of which had an Rf similar to thiamine and two which did not. Most of the radioactivity of the rechromatographed "thiamine spot" appeared at the thiamine location. Thus, no positive conclusion could be made, but the evidence does seem to point toward the existence of selenothiamine.

There is one documented instance of selenium replacing sulfur in a growth factor. Mautner and Günther (1959) replaced pantethine with its synthetic seleno-analog in the growth medium of Lactobacillus helveticus. The organism grew to a density almost equal to that obtained with pantethine.

Since selenopantethine supports growth it would seem that at least some of the enzymes of L. helveticus accept selenopantethine in place of its natural sulfur analog. In

fact, it has been discovered that certain enzymes, from various organisms, for which sulfur containing compounds are the natural substrate are also functional with the selenium analogs of these compounds. Among these enzymes is adenosine triphosphate sulfurylase which catalyzes the reaction of ATP with sulfate or selenate to form APS or APSe (Wilson and Bandurski, 1958). It is reported that selenomethionine is a better substrate than methionine for the enzyme responsible for the reaction of methionine and ATP to form S-adenosylmethionine (Mudd and Cantoni, 1957). Hoffman et al. (1970) showed that methionine and selenomethionine are used to about the same extent by methionyl aminoacyl-tRNA synthetase. Then in 1972 McConnell and Hoffman, using a cell free system obtained from E. coli, reported that selenomethionyl-tRNA can participate in protein synthesis in the same manner and to about the same extent as methionyl-tRNA and also that N-formyl selenomethionyl-tRNA can function as an initiator of protein synthesis in E. coli.

Thus, while it is clear that selenate, selenite and certain selenium analogs of sulfur compounds can penetrate sulfur metabolic pathways, make use of certain enzymes and form functional analogs of some other essential sulfur compounds, it is equally clear that selenium compounds undergo certain reactions different from those of sulfur compounds. Among the sulfur compounds for which no selenium analog has been found is 3'-phosphoadenosine-5'-phosphosulfate (PAPS)

(See review by Stadtman, 1973). In 1958 Wilson and Bandurski made an unsuccessful attempt to demonstrate PAPSe or its decomposition products in yeast provided with selenate.

Under normal conditions PAPS functions as a sulfate donor in the synthesis of sulfate esters such as heparin and chondroitin sulfate. Selenium analogs of these sulfate esters have not been demonstrated to occur in organisms grown with a high concentration of selenium (Stadtman, 1974; Allaway, 1973).

Taking a different approach to this problem Shrift and Virupaksha (1965) found that whereas glutathione was produced when selenium accumulating plants belonging to the genus Astragalus were supplied with $SO_4^=$, Se-gluthathione was not produced when $SeO_3^=$ or $SeO_4^=$ were supplied to the same organisms. Later the same year these authors reported that when two species of nonselenium accumulating Astragalus were supplied with H_2SeO_3, Se-methylselenocysteine accounted for a sizeable percentage of the seleno-amino acid produced; however, when the same organisms were supplied with $H_2{}^{35}SO_4$ in place of $H_2{}^{75}SeO_4$ no methylcysteine could be found (Virupaksha and Shrift, 1965). These studies and those demonstrating the enzymatic reduction of selenite by microorganisms (discussed previously) show that selenium does not always follow the sulfur metabolic pathways.

Biotin Synthesis

Biotin synthesis appears to occur by a four enzyme metabolic pathway common to all known biotin synthesizing organisms (see Fig. 1). Pimelic acid has been demonstrated as the starting substrate in this pathway by a number of studies in which ^{14}C-labeled pimelic acid was incorporated into biotin (Eisenberg, 1962; Elford and Wright, 1963; Eisenberg and Maseda, 1966). Some authors (Eisenberg and Star, 1968; Izumi et al., 1972) believe, however, that it is pimelyl-CoA rather than pimelic acid itself which participates in the synthesis. Izumi et al. (1972) coupled the formation of pimelyl Co-A from pimelic acid and CoA with the first reaction in the biotin synthetic pathway and thus demonstrated the formation of pimelyl-Co-A using cell free extracts of various bacteria. In the first reaction of the biotin pathway pimelyl-Co-A and L-alanine join to give 7-Keto-8-aminopelargonic acid (KAPA). In 1974 Izumi et al. reported that partially purified pimelyl-CoA synthetase, isolated from Bacillus megaterium, required ATP and Mg^{+2} as cofactors, was inhibited by metal-chelating agents and was not subject to feedback repression by biotin.

KAPA synthetase has been isolated from E. coli (Eisenberg and Star, 1968) and from Bacillus sphaericus (Izumi et al., 1973 b). It requires pyridoxal 5' phosphate (PLP) as a cofactor and pimelyl-CoA and alanine as substrates. No other amino acids were found to act as substrates though some of them were inhibitory. The KAPA so

Figure 1.

Abbreviations

PLP - pyridoxal 5' phosphate

KAPA - 7-keto-8-aminopelargonic acid

SAM - S-adenosyl-L-methionine

DAPA - 7,8-diaminopelargonic acid

DTB - desthiobiotin

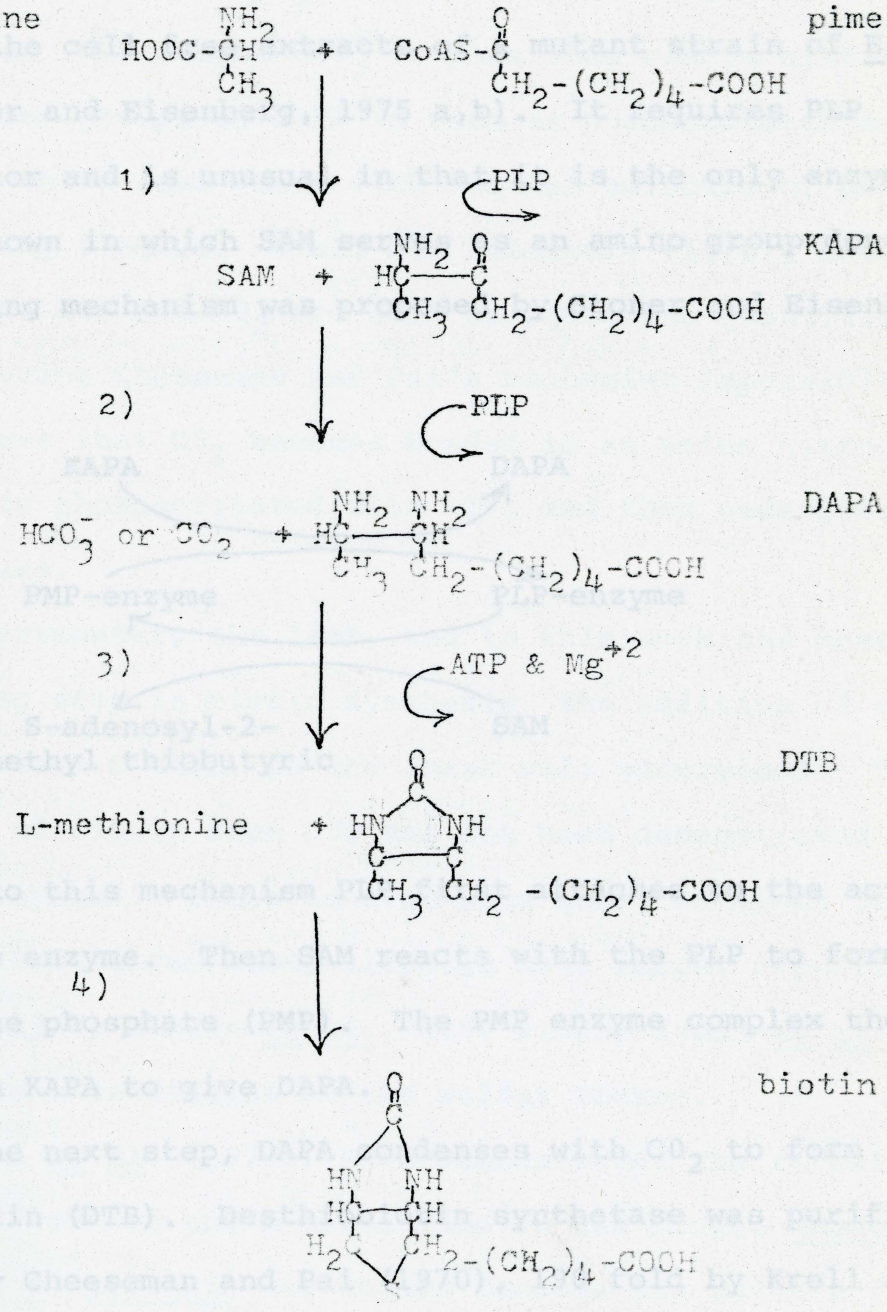
References

1) Eisenberg & Star, 1968
Izumi et al; 1973b

2) Stoner & Eisenberg, 1975 a,b

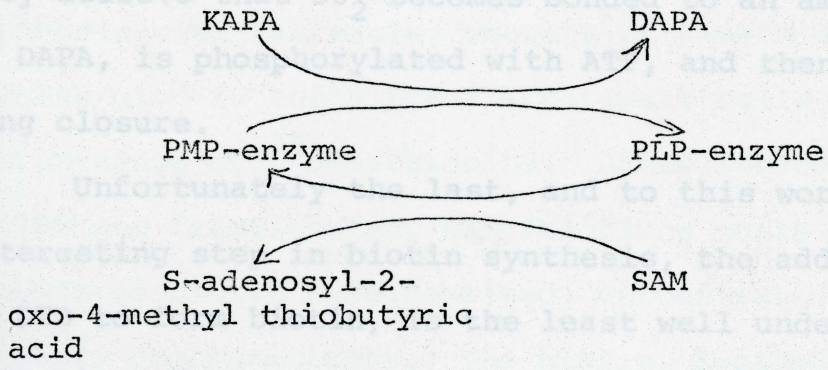
3) Cheeseman & Pai, 1970
Krell & Eisenberg, 1970

4) Nimura et al; 1964 a,b,c
Schimada, 1967
Izumi et al; 1973a


alanine
pimelyl CoA
1)
PLP
KAPA
SAM
2)
PLP
DAPA
HCO_3^- or CO_2
3)
ATP & Mg^{+2}
DTB
L-methionine
4)
biotin

formed is aminated to give 7,8-diaminopelargonic acid (DAPA).

KAPA aminotransferase, the enzyme that catalyzes the transfer of an amino group from S-adenosyl-L-methionine (SAM) to KAPA, thus forming DAPA, was purified almost 1,000 fold from the cell free extracts of a mutant strain of E. coli (Stoner and Eisenberg, 1975 a,b). It requires PLP as a cofactor and is unusual in that it is the only enzymatic reaction known in which SAM serves as an amino group donor. The following mechanism was proposed by Stoner and Eisenberg (1975 a,b).



According to this mechanism PLP first attaches to the active site of the enzyme. Then SAM reacts with the PLP to form pyridoxamine phosphate (PMP). The PMP enzyme complex then reacts with KAPA to give DAPA.

In the next step, DAPA condenses with CO_2 to form desthiobiotin (DTB). Desthiobiotin synthetase was purified 200 fold by Cheeseman and Pai (1970), 190 fold by Krell and Eisenberg (1970) and was also isolated by Yang et al. in 1971. All three groups agree that Mg^{+2} and ATP are required cofactors, but Krell and Eisenberg report that CO_2 is the

substrate while Cheeseman and Pai, and Yang et al. claim that HCO_3^- is the reactive species. Cheeseman and Pai (1970) state that if ^{14}C-HCO_3^-, is used in the reaction mixture the resulting DTB is labeled in the ureido carbon. They propose a mechanism in which HCO_3^-, activated by ATP, reacts with the enzyme. The enzyme HCO_3^- complex then reacts with one of the amino groups of DAPA to form an N-substituted monocarbonate which must react with another molecule of ATP to undergo ring closure. Krell and Eisenberg (1970), however, report that ATP and CO_2 are used in equimolar quantities thus rendering Cheeseman and Pai's mechanism impossible. They believe that CO_2 becomes bonded to an amino nitrogen of DAPA, is phosphorylated with ATP, and then undergoes ring closure.

Unfortunately the last, and to this work the most interesting step in biotin synthesis, the addition of sulfur to DTB to form biotin, is the least well understood. The formation of biotin from DTB has not been demonstrated in a cell free system. There is even some question about the source of the sulfur atom, although most recent reports (Shimada, 1967; Nimura et al., 1964 a,b,c; Izumi et al., 1973a) give L-methionine as the sulfur source.

Izumi et al. (1973a) have demonstrated the formation of biotin from DTB and L-methionine in the fungus Rhodotorula glutinis. R. glutinis was chosen because its culture filtrates were found to contain 300-400ng/ml of biotin. In the first experiment cells of R. glutinis were incubated

for 24 hours in a reaction medium which contained DTB and one of several sulfur sources along with other nutrients. At the end of the incubation period the amount of biotin was determined by microbiological assay. Of the fourteen sulfur sources tested only methionine and 2-mercaptoethanol, which is not present in cells, caused a substantial increase in biotin production over the control which contained no sulfur compound. (There was some biotin produced in all samples indicating that there was growth and that 24 hours is not sufficient time to cause death due to sulfur starvation in this organism). They were able to show increased biotin synthesis with increasing amounts of L-methionine up to 50 µmoles/ml. In their final experiment cells were grown in the presence of ^{35}S-methionine. After incubation, the medium was fractionated, and fractions containing significant amounts of biotin were combined, concentrated and subjected to paper chromatography. The biotin, located by bioautography, occurred coincident with a peak of radioactivity. Thus it appears that methionine is the sulfur source for biotin. More conclusive evidence must await the isolation of "biotin synthetase".

Evidence that biotin synthesis in Phycomyces blakesleeanus takes place by these four steps has been provided by the work of Eisenberg (1963) and Chenouda and El-Awamry (1971a,b,c). Eisenberg found the following biotin vitamers in the culture medium of P. blakesleeanus: biotin, DTB, biotin-d-sulfoxide, biocytin (biocytin is a biologically

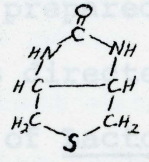
active form of biotin, in which the carboxyl group of biotin is bound to an amino group of an epsilon lysine in a peptide chain) and an unidentified compound believed to be a pelargonic acid derivative. Chenounda and El-Awarmy also found biotin, DTB, biotin-d-sulfoxide and biocytin in the medium of P. blakesleeanus. In addition they also found KAPA and DAPA. Birnbaum and Pai (1967) showed that biotin sulfoxide is an oxidation product of biotin and probably not biological in origin. Thus, even though none of the biotin synthetic enzymes has been isolated in P. blakesleeanus it has been shown to produce KAPA, DAPA and DTB, the intermediates in the common biotin synthetic pathway. The presence of these intermediates is reasonably good evidence that P. blakesleeanus has the same enzymes making the same intermediates as do the organisms which have been used in the study of the synthesis of biotin.

Reasons for Expecting the Isolation of Selenobiotin

There are some observations which point to the possible biosynthesis of a selenobiotin. Since methionine appears to be the sulfur source for biotin, presumably selenomethionine would be the selenium source for selenobiotin. This is interesting because formation of selenomethionine by various organisms is well documented (Tuve and Williams, 1961; Huber et al., 1967). In addition other authors have demonstrated that selenomethionine can replace methionine

as a substrate for several enzymes (Mudd and Cantoni, 1957; Hoffman et al., 1970). Thus, it would appear that P. blakesleeanus, when supplied with selenite or selenate, might synthesize selenomethionine and that "biotin synthetase" might be able to use selenomethionine in place of methionine thus producing selenobiotin from DTB and selenomethionine.

In addition, there is evidence that selenobiotin so formed would be stable enough to be identified by bioautography. (Martin (1975) succeeded in synthesizing cis-3,4-ureyleneselenophane demonstrating that selenium can replace



sulfur in a relatively stable thio ether structure.) Shrift (1967) published preliminary evidence for the biosynthesis of selenothiamine which has a cyclic thio ether structure similar to biotin. He reported evidence for the synthesis of selenothiamine by E. coli.

More recently Bory and Marquet (1976) have reported the chemical synthesis of selenobiotin. They report that their selenobiotin has a conformation similar to that of biotin even though the length of the C-Se bond is somewhat greater than the length of the C-S bond (1.96 and 1.82 Å respectively). The biochemical properties of this compound have yet to be explored.

MATERIALS AND METHODS

Media

All media were prepared with distilled water and sterilized by autoclaving at 121°C for 15 minutes unless otherwise stated.

The following prepared media were obtained from Difco and reconstituted as directed: Micro Inoculum agar, used for the maintenance of *Lactobacillus plantarum*; Sabouraud agar, slants of which were used for the maintenance of *Saccharomyces cerevisiae* and *P. blakesleeanus*; and biotin assay medium, which was used in the biotin assay with *L. plantarum*. Biotin assay medium was sterilized for only 7 minutes at 121°C.

The yeast culture medium used in the *S. cerevisiae* biotin assay was prepared by reconstituting Difco vitamin free yeast base at a concentration ten times that used in culture. To 100 ml of concentrate, 1 mg thiamine, 1 mg. pyridoxine and 0.05 mg calcium pantothenate were added (Hertz, 1943). The concentrate was then sterilized by passage through a membrane filter and frozen until used. Yeast base was prepared for inoculation by the aseptic addition of 10% (V/V) yeast base concentrate to sterile water.

Eisenberg's (1963) culture medium for P. blakesleeanus, modified by addition of pimelic acid and by the substitution of urea for L-asparagine (Chenouda and El-Awamry 1971b), was used for biotin production. This medium was composed of the following ingredients:

glucose	30g
urea	1.4g
KH_2PO_4	1.5g
$MgSO_4 \cdot H_2O$	0.5g
Thiamine HCl	100 mg
pimelic acid	0.5g
ferric citrate sol.	0.1 ml
trace elements sol.	0.1 ml

dissolved in sufficient water to give a final volume of 1 liter. As recommended by Eisenberg, the trace element mixture of Hammer et al (1943) was used. This trace element mixture consists of two solutions, one of ferric citrate and the other of the remaining salts. The ferric citrate solution was prepared by adding 1.80g of ferric sulfate and 1.89g of citric acid monohydrate to one liter of water. The trace element mixture had the following composition:

$ZnSO_4 \cdot 7H_2O$	0.46g
$CuSO_4 \cdot 5H_2O$	0.078g
$MnSO_4 \cdot H_2O$	0.31g
$(NH_4)_2MoO_4$	0.104g
H_3BO_3	0.0572g

and sufficient water to make a total volume of 1 liter. The pH of the medium was adjusted to 4.5 with NaOH (Chenouda and El-Awamry, 1971a; Eisenberg, 1963).

Biotin Assay

Biotin concentrations were measured by microbiological assay with either Lactobacillus plantarum (ATCC 8014) or Saccharomyes cerevisiae (ATCC 9896). Both of these organisms require biotin for growth; however, L. plantarum will respond only to biotin or biotin sulfoxide among the naturally occurring biotin vitamers (Waller and Lichstein, 1965), whereas S. cerevisiae will utilize DTB, KAP and DAP in addition to biotin and biotin sulfoxide. Thus, the L. plantarum assay quantifies the amount of "true biotin" and S. cerevisiae measures the amount of "total biotin" (Sebrell and Harris, 1968). Two different procedures were employed with each organism.

Procedure 1, which related the amount of growth in a culture tube to the concentration of biotin in that tube, was a quantitative method. Standards which contained carefully measured amounts of biotin were run in triplicate, providing a reasonably accurate standard curve for biotin concentration. Culture tubes were selected to fit a Klett-Summerson colorimeter. The turbidity of each tube was measured with the colorimeter using filter no. 66 just after inoculation and again after the incubation period. Subtracting the initial from the final readings corrected for differences in the tubes or variability in the color of the medium.

Procedure 2 was used when a quick test for the presence

of biotin was needed. In this case pieces of filter paper impregnated with a sample were placed on the surface of a biotin-free agar plate seeded with S. cerevisiae or L. plantarum. Growth around the filter paper indicated the presence of biotin.

L. plantarum assays.

L. plantarum was maintained in micro inoculum agar stabs. After inoculation the stabs were incubated at 37°C for two days and then refrigerated until used.

Procedure 1 tube assay: This quantitative biotin assay is based on a method published in 1974 by Bell. A twenty-four hour culture of L. plantarum, grown in Micro Inoculum broth, served to inoculate ten ml of biotin-free biotin assay medium which was incubated for 20 hours at 37°C and then used as inoculum for this assay.

Culture tubes used in this procedure must be scrupulously clean. Even traces of detergent may produce a nonspecific stimulation (Waller, 1970). Each tube contained 2.5 ml of double strength biotin assay medium, a measured amount of either a biotin solution or of a sample being assayed, and sufficient water to make a total volume of 5 ml.

The six standards which were run in triplicate contained 0.005, 0.010, 0.015, 0.020, 0.025 and 0.05 ng of biotin per ml. In addition to three blanks which were

inoculated, an uninoculated tube, which served to zero the Klett, was prepared.

After the assay medium was prepared, it was sterilized at 121°C for 7 minutes. After inoculation with one drop of the L. plantarum inoculum, and the assay tubes were incubated for 48 hours.

The values obtained for the standards 0.00 - 0.025 ng biotin/ml were used to prepare a standard curve by a least squares plot from which the amount of biotin in the various samples was estimated.

Procedure 2 plate assay: The L. plantarum inoculum for this procedure was cultured for 24 hours at 37°C in ten ml of biotin assay medium which contained 0.2 ng of biotin (Genghofe et al., 1948). The cells were harvested by centrifugation, washed twice in 0.9% saline and then re-suspended in 3 ml of 0.9% saline. About 0.6 ml of the cell suspension was added to every 50 ml of biotin assay agar.

Biotin assay agar was prepared by adding 1.5% agar to biotin assay medium in flasks and autoclaving at 121°C for 7 minutes. After sterilization, the flasks were kept in a water bath at 47°C for 30 minutes to insure cooling. The inoculum was added, and then the agar medium was poured into petri dishes and allowed to harden. These plates had to be used within three days. Older plates do not always show growth in response to biotin, presumably due to the death of L. plantarum.

Pieces of Whatman No. 1 filter paper were dipped into

the samples to be tested, into standards with known concentrations of biotin, and into water blanks, and then placed on the surface of the seeded agar plates. Assay plates were incubated at 37°C for at least 24 hours. After incubation the width of the growth zone around the filter paper was measured.

S. cerevisiae assays.

S. cerevisiae was maintained on Sabouraud agar slants. After transfer, cultures were incubated at room temperature for about three days until a heavy white growth developed. They were stored in a refrigerator. One loop of S. cerevisiae obtained from an agar slant was suspended in 3 ml of 0.9% saline. The suspension was centrifuged, the supernatant was decanted, and the cells were resuspended in 3 ml of saline. This washing process was repeated two more times in order to prepare an inoculum. The washed cell suspension was used full strength in procedure two, but was diluted by adding 0.1 ml of cell suspension to 3 ml of saline for use in procedure one.

Procedure 1, the tube assay: The method of Hertz (1943) was used as a reference for the assay described below; however, the materials and equipment available necessitated radical modification.

As with the L. plantarum assay, the standards which contained 0.005, 0.010, 0.015, 0.020, 0.025 and 0.05 ng of

biotin per ml and the inoculated blanks were run in triplicate. An uninoculated tube served to zero the Klett. Culture tubes containing biotin or sample to be assayed and sufficient water to give a volume of 4.5 ml were autoclaved at 121°C for 15 minutes and allowed to cool. Once cool, 0.5 ml of concentrated yeast culture medium and one drop of dilute S. cerevisiae inoculum were added to each tube. The assay tubes were incubated for 4 days at room temperature. A least squares plot provided a standard curve from which biotin concentrations of various samples were estimated.

Procedure 2, plate assay: Water containing 1.5% agar was autoclaved at 121°C for 15 minutes and then cooled in a 47°C water bath for at least 30 minutes. When cool, 10% (V/V) yeast base and 0.5 ml of undiluted inoculum for every 50 ml of water were added. The medium was poured into sterile petri dishes and allowed to harden. Pieces of Whatman No. 1 filter paper were dipped into the samples being tested, drained and placed on the surface of the needed plates. Plates were incubated at room temperature for at least 24 hours, after which the width of the growth zone around the paper was measured. Plates remained usable for at least one week when stored in the refrigerator.

Measurement of Radioactivity

[^{35}S]-sulfur was counted on the ^{14}C channel of a Packard liquid

scintillation spectrometer model No. 3375. Bray's solution (Bray, 1960) was used as cocktail because the samples being counted were in an aqueous solution. One tenth ml of sample was counted in 10 ml of Bray's solution.

[^{75}Se]-selenium was counted in 1 ml liquid aliquots in a Packard gamma scintillation spectrometer model No. 5320.

Paper chromatograms were scanned for radioactivity in a Packard radiochromatogram scanner model No. 7201. After scanning, chromatograms containing ^{75}Se were cut cross-wise into 1 cm strips. The strips were folded and placed in tubes to be counted in the Packard gamma scintillation spectrometer.

Culture of Phycomyces blakesleeanus (ATCC 6200)

P. blakesleeanus was maintained on Sabourand slants (Eisenberg, 1963). To prepare a spore suspension, 2 to 4 ml of sterile water were pipetted onto the surface of an agar slant. The spores were loosened with a Vortex Jr. mixer and then activated at 50° C for 15 minutes (Eisenberg, 1963). To maintain the culture, a small amount of spore suspension was pipetted onto the surface of a new slant and allowed to grow for about 5 days at room temperature until black sporangia were present. The slant could be refrigerated for several weeks before use.

Flasks of liquid biotin production medium were inoculated with the spore suspension. Inoculated flasks

were placed in a shaking water bath at 30°C for eight days, since both Eisenberg (1963) and Chenouda and El-Awamry (1971a) demonstrated that the amount of biotin reaches a maximum in that time.

Ion Exchange Chromatography

Two ion exchange resins, both obtained from Bio-Rad, were used to prepare a biotin concentrate from P. blakesleeanus medium. After the growth period, the mycelium was removed from the medium and the pH of the medium was adjusted to 2. The medium was then passed through an AG-50w x 8 H^+ column (Eisenberg and Maseda, 1966; Izumi et al. 1973a). The column was washed with water until no more biotin could be detected with the L. plantarum or yeast plate assays. The radioactivity of representative samples was measured and the biotin-containing fractions were combined and concentrated in a vacuum evaporator. The pH of the concentrate was adjusted to 7 before it was applied to an Ag-1 x 8 formate column (Eisenberg and Krell, 1969; Ogata, 1970; Izumi et al. 1973a). After application of the concentrate the column was washed with 100 ml of water and then with 0.012 M formic acid. The radioactivity of representative fractions was measured. Biotin was located by the L. plantarum plate assay, and biotin-containing fractions were combined and concentrated to a few ml in preparation for paper chromatography.

Emergence Patterns of Biotin Vitamers from Ion Exchange Columns

AG-50: When P. blakesleeanus medium is placed on a Dowex 50 H^+ column, biotin, DTB and biotin-d-sulfoxide are found in the water wash. Other biotin vitamers are retained by the column (Eisenberg, 1963).

AG-1: Ogata (1970), was able to obtain large amounts of biotin vitamers from the culture medium of Bacillus sphaericus IF03225. Upon application of the B. sphaericus medium to a Dowex-1-formate column, Ogata reported that DAPA was found in the water wash and that biotin-d-sulfoxide, DTB and biotin emerged in that order when the column was developed with 0.012 M formate. Chenouda and El-Awamry (1971a) reported a similar emergence pattern from a De Acidite FF (formate) column (Koch-Light Laboratories) for biotin vitamers produced by P. blakesleeanus.

In light of this information biotin vitamers were located in the eluate of an AG-1 column by both the L. plantarum and yeast assays. Thus, a vitamer in the water wash active for yeast but inactive for L. plantarum could tentatively be identified as DAPA. Three vitamers should be eluted with 0.012 M formic acid. First, biotin-d-sulfoxide active with L. plantarum and yeast should emerge. Next, DTB active only with yeast should appear; and lastly, biotin active with either L. plantarum or yeast should emerge from the column.

Paper Chromatography

Descending paper chromatography was carried out at room temperature with n-butanol: water: glacial acetic acid (4:5:1) as the solvent (Chenouda and El-Awamry 1971a). This solvent separates into two layers, the upper phase is used as the solvent, whereas the lower phase is used to saturate the atmosphere of the chromatocab (Wright et al. 1954). In all cases the sample was applied to Whatman No. 1 paper as a band. Chromatograms were run about 12 hours. They usually reached a length of about 30 cm.

Bioautography

Bioautography is a technique in which microbiological assay is used to develop a paper chromatogram. In this case growth of L. plantarum was used to indicate the presence of biotin. Pyrex dishes 20 x 35 cm were fitted with aluminum foil tops and sterilized. One hundred ml portions of biotin assay medium with 1.5g of agar were autoclaved for 7 minutes at 121°C. Upon removal from the autoclave the biotin assay agar was kept at 47°C for at least 30 minutes to insure cooling. The L. plantarum innoculum was prepared in exactly the same way as for the biotin plate assay. After the cooling period, 1.2 ml of L. plantarum cells were thoroughly mixed into the 100 ml biotin assay agar which was then poured into a sterile pyrex dish so that the bottom

of the dish was covered with an even layer of agar.

After the agar had hardened, strips not more than 1 cm wide were cut length-wise from the chromatogram, sprayed with water so that they were damp, but not dripping, and placed on the surface of the agar. Normally, a paper strip was allowed to remain on the agar for one minute and then moved to a new location in the same agar dish. A strip was usually moved 4 to 5 times and allowed to remain in its final position for the entire incubation period. Biotin diffuses rapidly from these wet paper strips. Since the chromatograms generally contained many times the quantity of biotin needed to support growth of L. plantarum, moving the strips provided a series of bioautograms each containing less biotin than the one before. The bioautogram showing the smallest but still clearly visible area of growth was chosen for measurement of Rf values. These plates were incubated about 20 hours at 37°C. White opaque areas of growth indicated the location of biotin on the chromatograms.

Isolation of Biotin (Selenobiotin)

Since the purpose of this research was to determine if P. blakesleeanus could synthesize biotin in which the sulfur atom was replaced by selenium, the general plan was to allow this organism to grow in the presence of radioactive selenite or selenate and then to determine if the biotin produced was radioactive. Because P. blakesleeanus excretes about

90% of the biotin vitamers it produces (Eisenberg, 1963), it was possible to recover biotin from the growth medium thus eliminating drastic procedures, e. g. boiling in 6N H_2SO_4 which are necessary to extract biotin from tissues.

Therefore, the mycelia were removed from the medium after the growth period, and the media were passed through two ion exchange columns as described above. The concentrate from the AG-1 column was used to prepare paper chromatograms, which were scanned for radioactivity, subjected to bioautography and in the case where they contained ^{75}Se cut into strips to be counted in the gamma scintillation counter according to the methods described above.

Five batches of P. blakesleeanus medium were investigated in this manner. One batch of medium was labeled $^{35}SO_4^{=}$. Two other batches were labeled with $^{75}SeO_3^{=}$ and the last two had $^{75}SeO_4^{=}$ as a marker.

Possible Volatilization of Selenite by P. blakesleeanus

The results of some experiments indicated that selenite might be converted to volatile compounds by P. blakesleeanus. The following experiment was intended to explore the possiblity that selenite was volatilized. Three batches of 120 ml and one batch of 60 ml of P. blakesleeanus medium were prepared. A 3.0 mM stock solution of sodium selenite with 75µci of [^{75}Se] selenite per ml was prepared. A sufficient amount of the stock solution was added to each

of the 120 ml batches to give final selenite concentrations of 0.005, 0.010 and 0.020 mM. Enough stock was added to the 60 ml batch to give a selenite concentration of 0.040 mM. The medium was dispensed to give 36 ml per 125 ml Eylenmeyer flask, sterilized, inoculated and incubated in a shaking water bath at 30°C for 8 days. After growth, the mycelia were collected by filtration through membrane filters. The mycelia were dried at room temperature for two days in preweighed glass dishes, after which they were weighed again; approximately 50 mg were removed to be digested in 0.5 ml of concentrated HNO_3 and 0.5 ml of concentrated H_2SO_4 for 24 hours in preparation for measuring the radioactivity. The radioactivity and the biotin content of the media were also determined.

RESULTS

Identification of ^{35}S-labeled Biotin from the medium of P. blakesleeanus grown with ^{35}S-sulfate

The purpose of this experiment was to demonstrate ^{35}S-labeled biotin in the medium of P. blakesleeanus grown with ^{35}S-sulfate. This experiment was to serve as a model for and a standard of comparison with similar experiments in which ^{35}S-sulfate was replaced with ^{75}Se-selenate. The methods by which P. blakesleeanus was grown and by which biotin was isolated from the growth medium have been described previously (see Materials and Methods). Figure 2 gives the elution pattern of the medium from the AG-50 column. It is apparent that biotin, eluted as broad peak, is somewhat retarded by the resin. There does not appear to be any separation of biotin and DTB.

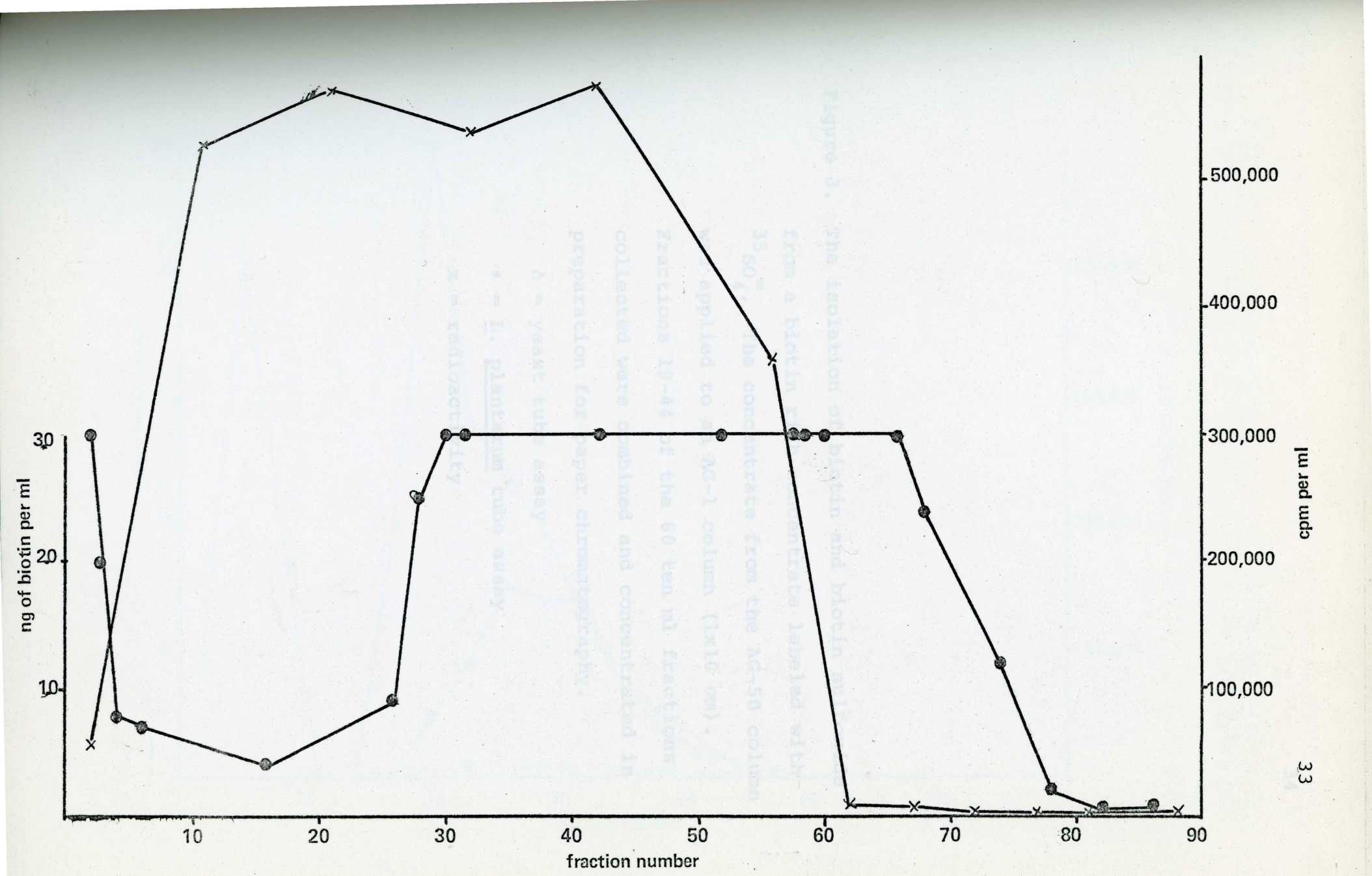
The elution pattern for the AG-1 column is shown in figure 3. Both Ogota (1970) and Chenouda and El-Awarmy (1971a) show a much sharper separation of biotin and DTB.

Nonetheless a radioactive biotin rich concentrate was obtained upon concentration of the biotin active fractions. The contamination of the biotin concentrate with DTB should not have affected the subsequent results

Figure 2. The separation of biotin active material by AG-50 column chromatography from medium in which P. blakesleeanus was grown with $^{35}SO_4^{=}$. Three hundred and fifty ml of medium with 350 µCi of $^{35}SO_4^{=}$ were dispensed into fourteen 125 ml Erylenmyer flasks, inoculated with P. blakesleeanus and incubated in a shaking water bath at 30°C for 8 days. The 324 ml of medium collected by filtration had 2 µg of biotin and 9 x 10^{10} counts/min of radioactivity. The filtered medium was passed through an AG-50 column (1x15 cm). Seventy-five 10 ml fractions were collected. Those fractions containing at least 1 ng/ml of biotin were combined and concentrated in preparation for the AG-1 column.

x = radioactivity

• = L. plantarum tube assay.


ng of biotin per ml
30
20
10
cpm per ml
500,000
400,000
300,000
200,000
100,000
10
20
30
40
50
60
70
80
90
fraction number

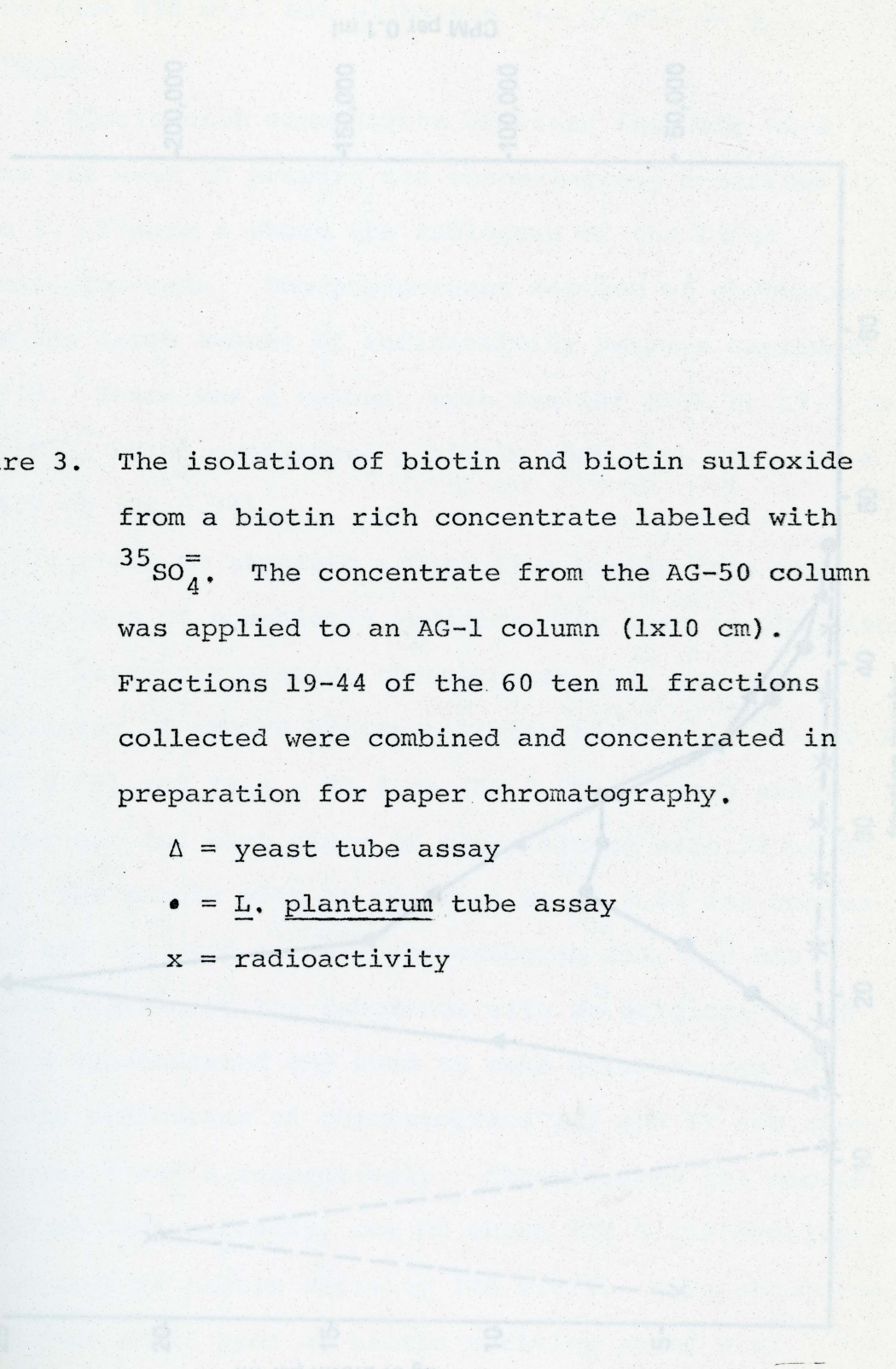
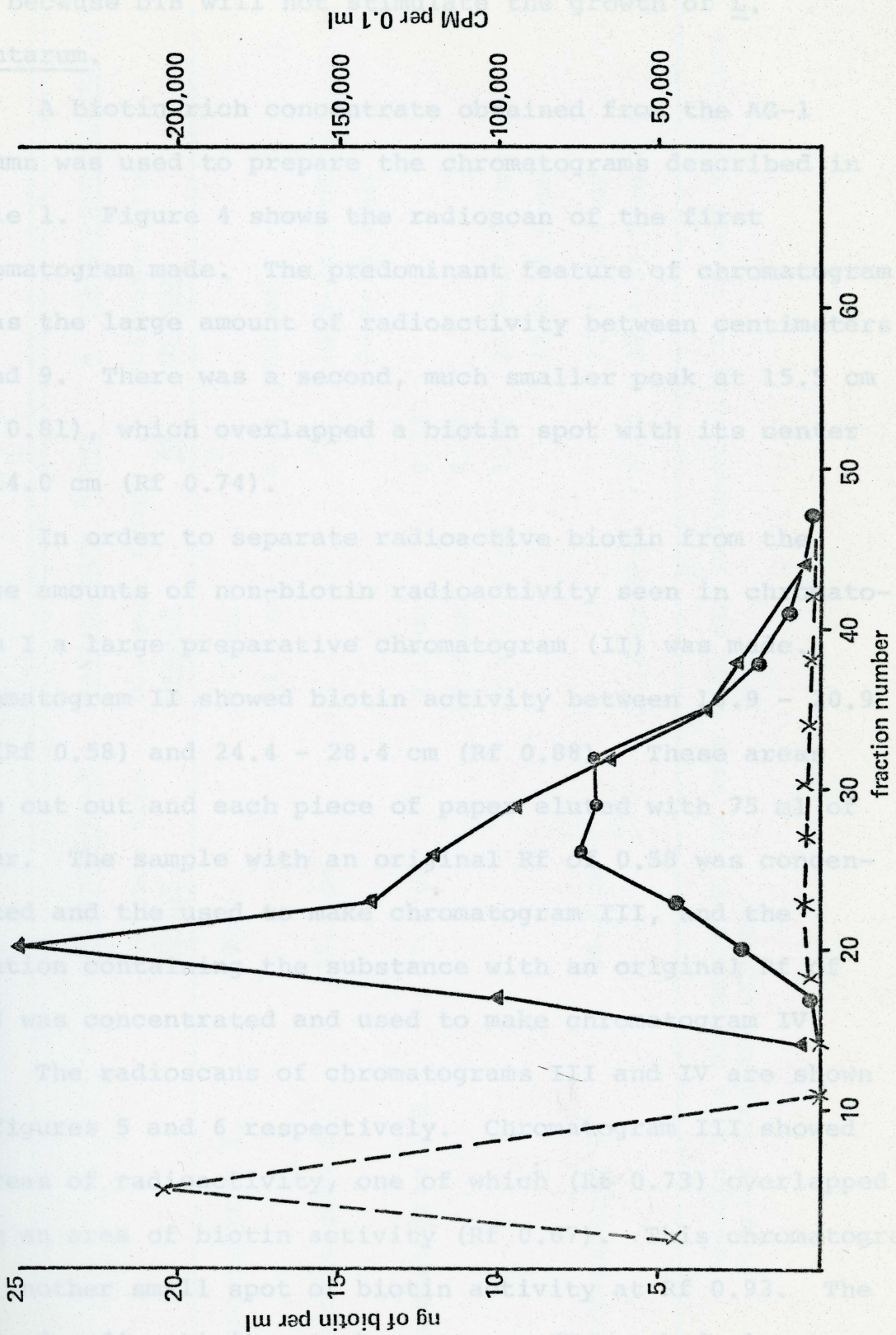


Figure 3. The isolation of biotin and biotin sulfoxide from a biotin rich concentrate labeled with $^{35}SO_4^{=}$. The concentrate from the AG-50 column was applied to an AG-1 column (1x10 cm). Fractions 19-44 of the 60 ten ml fractions collected were combined and concentrated in preparation for paper chromatography.

Δ = yeast tube assay

• = L. plantarum tube assay

x = radioactivity


CPM per 0.1 ml
200,000
150,000
100,000
50,000
ng of biotin per ml
25
20
15
10
5
fraction number
10
20
30
40
50
60

because, DTB lacking the S atom could not be radioactive and because DTB will not stimulate the growth of L. plantarum.

A biotin rich concentrate obtained from the AG-1 column was used to prepare the chromatograms described in table 1. Figure 4 shows the radioscan of the first chromatogram made. The predominant feature of chromatogram I was the large amount of radioactivity between centimeters 2 and 9. There was a second, much smaller peak at 15.5 cm (Rf 0.81), which overlapped a biotin spot with its center at 14.0 cm (Rf 0.74).

In order to separate radioactive biotin from the large amounts of non-biotin radioactivity seen in chromatogram I a large preparative chromatogram (II) was made. Chromatogram II showed biotin activity between 16.9 - 20.9 cm (Rf 0.58) and 24.4 - 28.4 cm (Rf 0.88). These areas were cut out and each piece of paper eluted with 75 ml of water. The sample with an original Rf of 0.58 was concentrated and the used to make chromatogram III, and the solution containing the substance with an original Rf of 0.88 was concentrated and used to make chromatogram IV.

The radioscans of chromatograms III and IV are shown in figures 5 and 6 respectively. Chromatogram III showed 3 areas of radioactivity, one of which (Rf 0.73) overlapped with an area of biotin activity (Rf 0.67). This chromatogram had another small spot of biotin activity at Rf 0.93. The area of radioactivity on chromatogram IV matched almost

Table 1 Rf values of biotin vitamers obtained by paper chromatography of the medium from *P. blakesleeanus* grown with $^{35}SO_4^{=}$.

	Solvent Front	Radioactive Areas		L. plantarum growth areas	
	Distance from origin	Distance from origin	Rf.	Distance from origin	Rf.
Chromatogram I.	19 cm	2-9 cm			
		15.5 cm	0.81	14 cm	0.74
Chromatogram II.	33.4 cm	*		16.9-20.9 cm	0.58
				24.4-28.4 cm	0.88
Chromatogram III.	37.8 cm	9.5 cm	0.25		
		13.0 cm	0.35		
		25.2-27.8 cm	0.73	23.7-27.3 cm	0.67
				32.4-34.8 cm	0.88
Chromatogram IV	38.4 cm			23.9-26.4 cm	0.66
		32.2-34.8 cm	0.88	32.4-34.8 cm	0.88

*Because this chromatogram was very wide the radioactivity on it was too dilute to be located with the scanner.

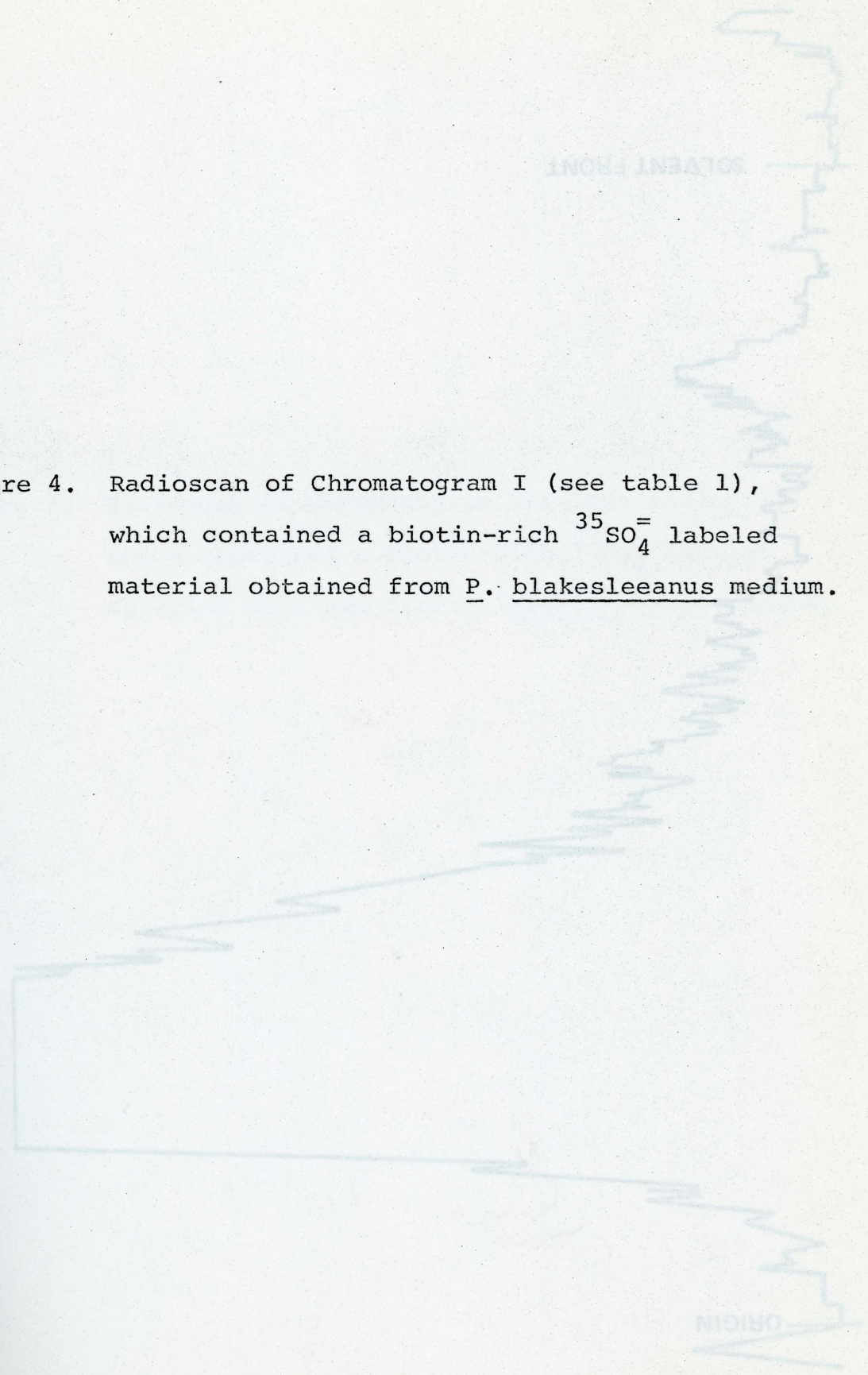
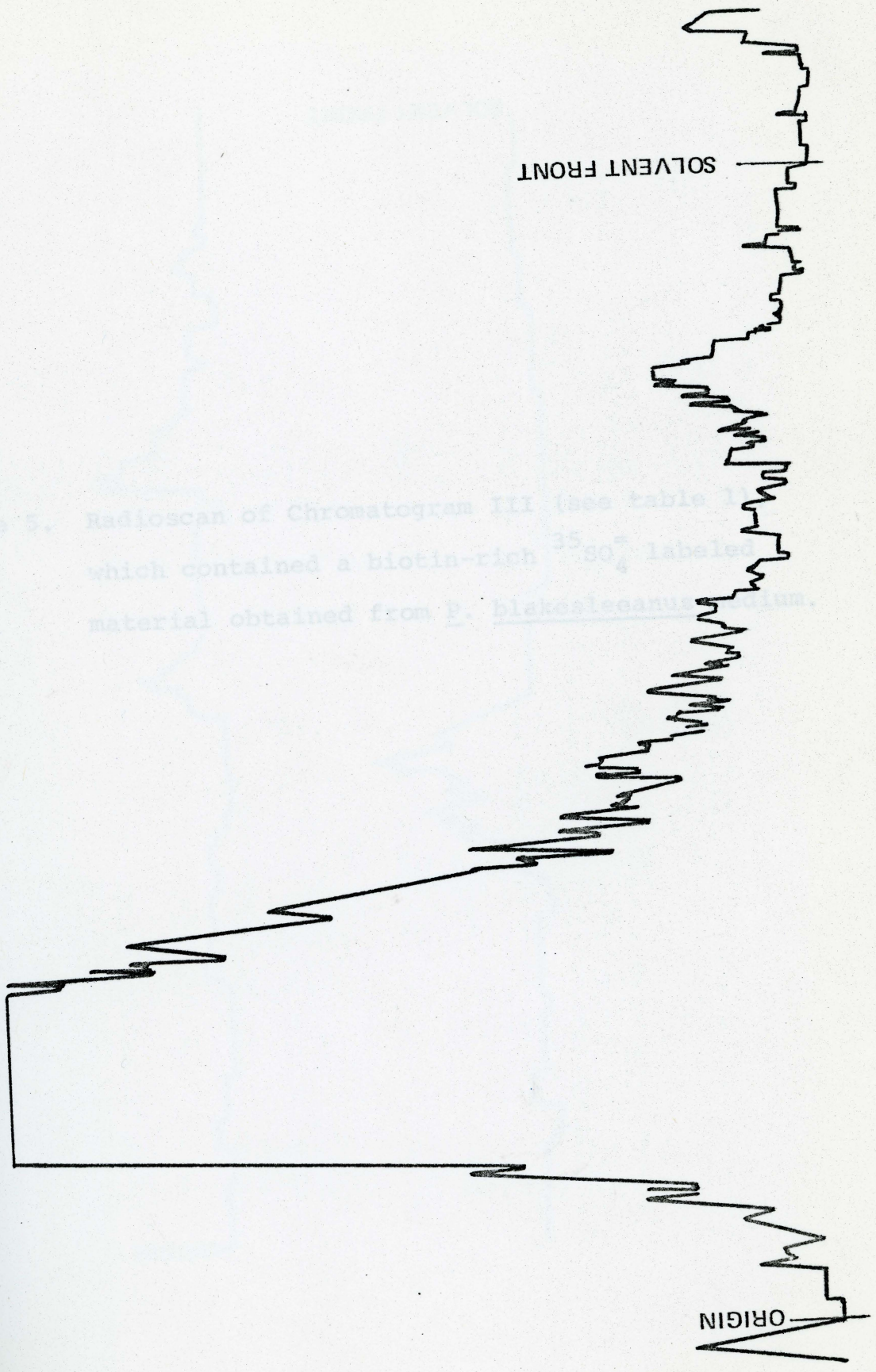


Figure 4. Radioscan of Chromatogram I (see table 1), which contained a biotin-rich $^{35}SO_4^=$ labeled material obtained from P. blakesleeanus medium.


SOLVENT FRONT
ORIGIN

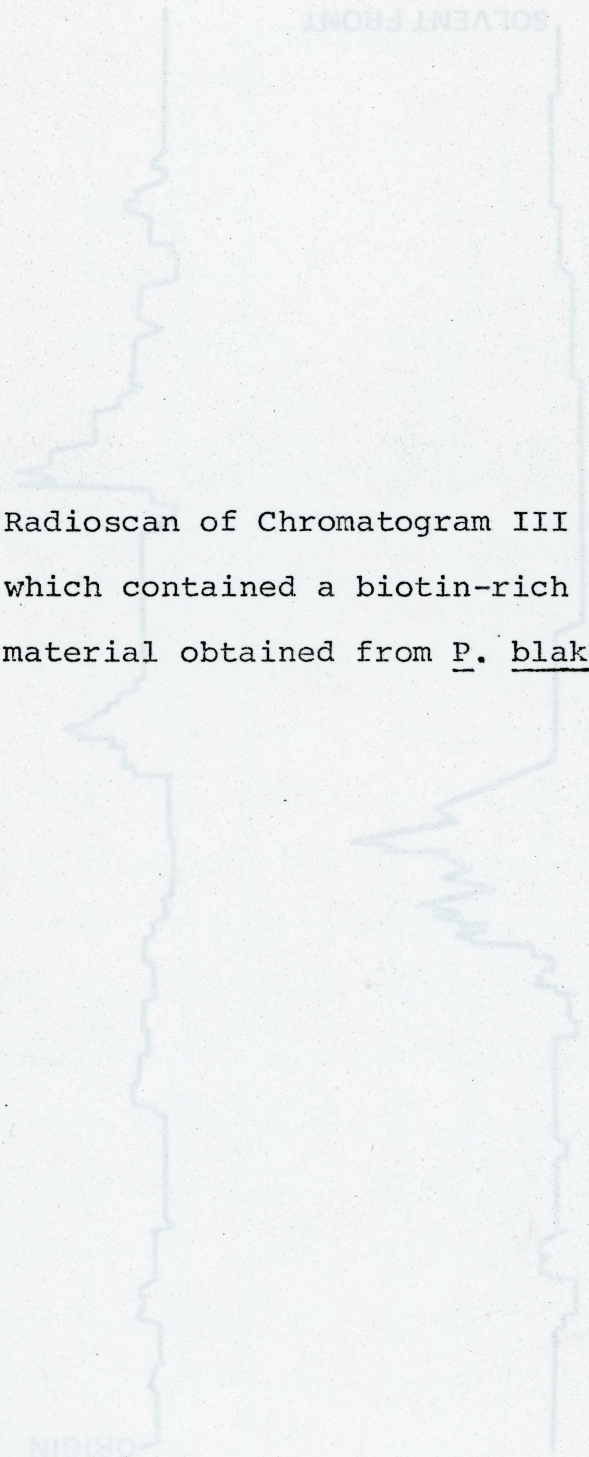
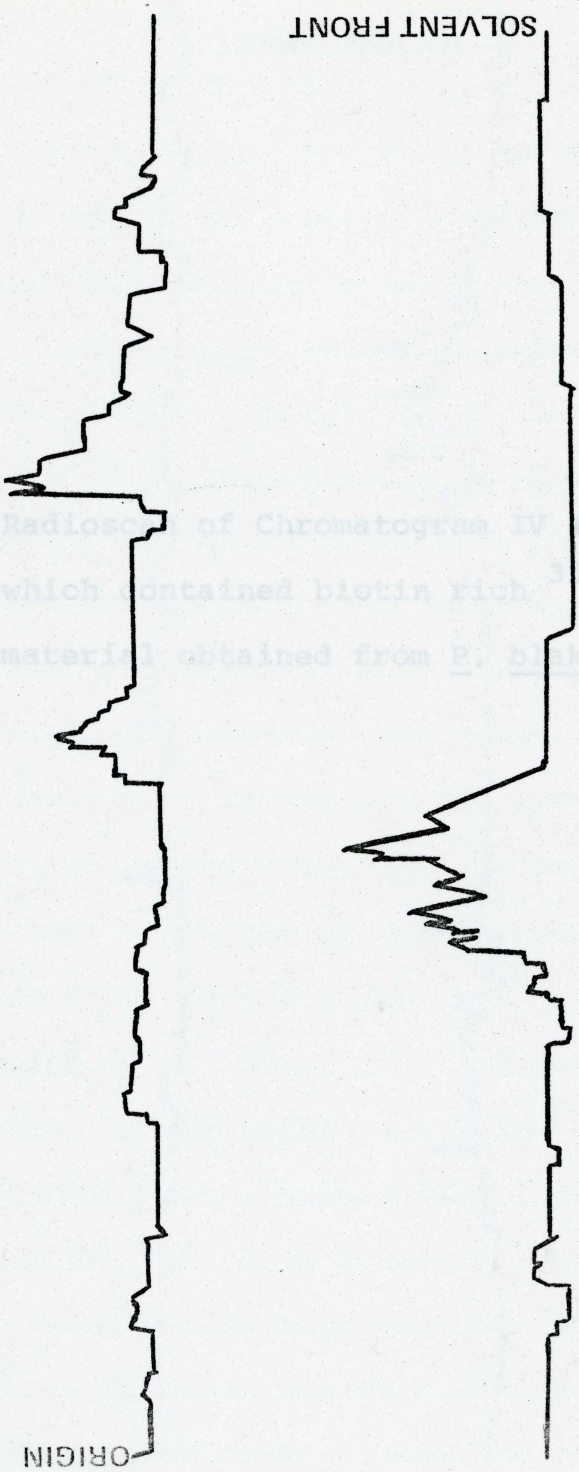


Figure 5. Radioscan of Chromatogram III (see table 1), which contained a biotin-rich $^{35}SO_4^=$ labeled material obtained from P. blakesleeanus medium.


SOLVENT FRONT
ORIGIN

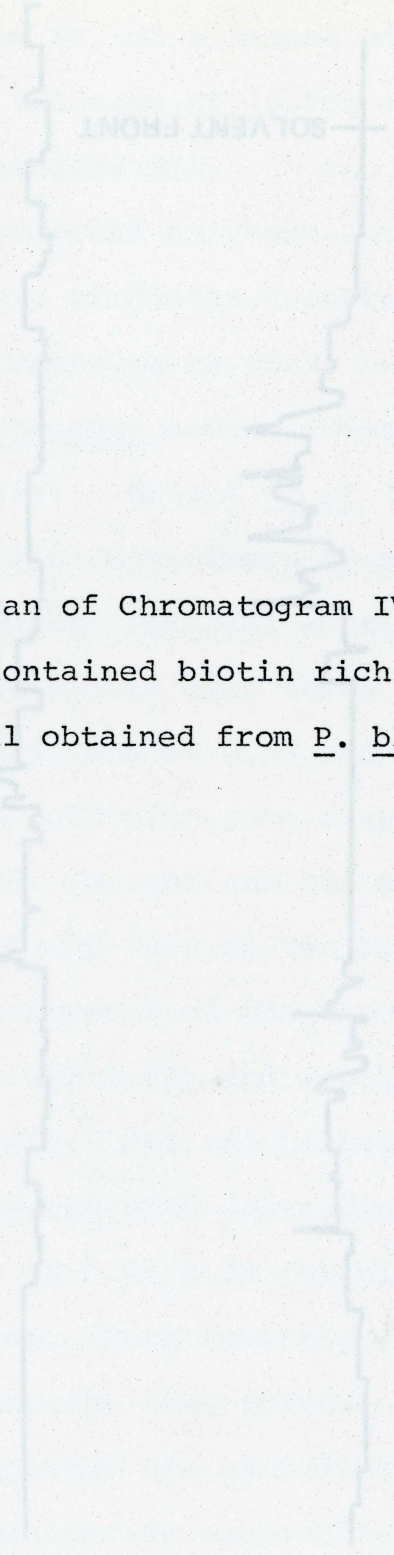
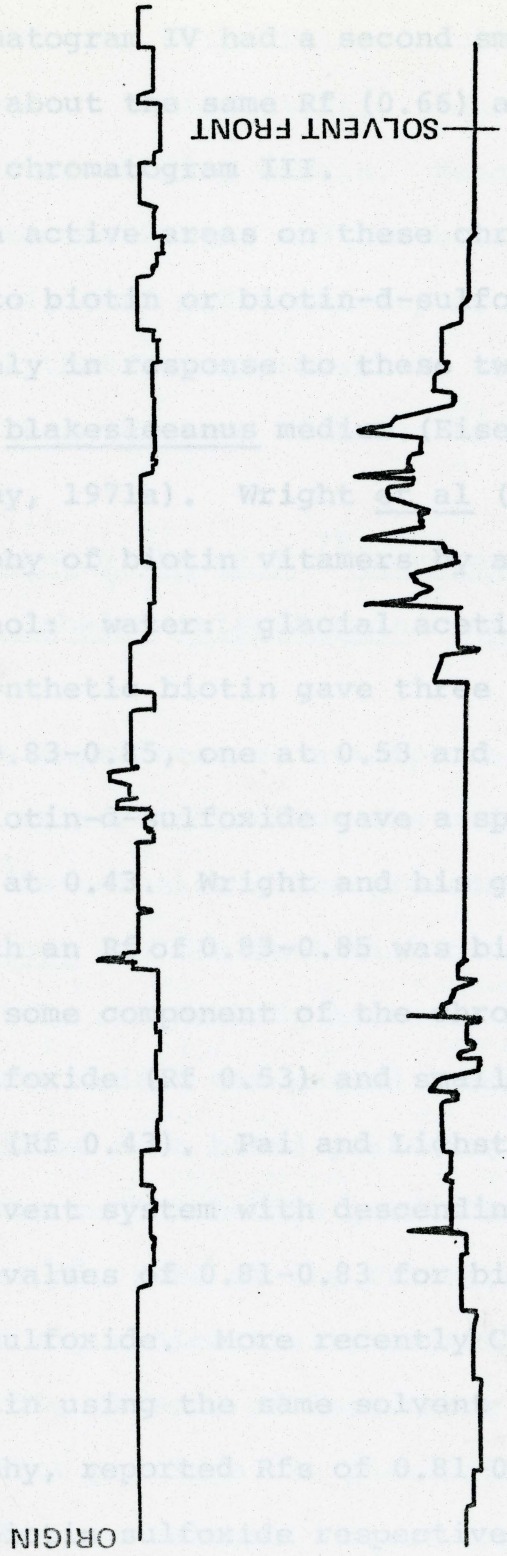


Figure 6. Radioscan of Chromatogram IV (see table 1), which contained biotin rich $^{35}SO_4^{=}$ labeled material obtained from P. blakesleeanus medium.


SOLVENT FRONT
ORIGIN

exactly an area of biotin activity. They both had Rfs of 0.88. Chromatogram IV had a second small area of biotin activity at about the same Rf (0.66) as the area of biotin activity on chromatogram III.

Biotin active areas on these chromatograms must correspond to biotin or biotin-d-sulfoxide since L. plantarum will grow only in response to these two vitamers among those found in P. blakesleeanus medium (Eisenberg, 1963; Chenouda and El-Awarmy, 1971a). Wright et al (1954) studied the chromatography of biotin vitamers by ascending chromatography with n-butanol: water: glacial acetic acid (4:5:1) as the solvent. Synthetic biotin gave three spots, a large one between Rf 0.83-0.85, one at 0.53 and a small one at 0.43. Synthetic biotin-d-sulfoxide gave a spot at Rf 0.57 and a smaller one at 0.43. Wright and his group concluded that the spot with an Rf of 0.83-0.85 was biotin, which was oxidized by some component of the chromatographic system to biotin-d-sulfoxide (Rf 0.53) and small quantities of biotin-l-sulfoxide (Rf 0.43). Pai and Lichstein (1965), who used the same solvent system with descending chromatography, reported Rf values of 0.81-0.83 for biotin and 0.55-0.58 for biotin sulfoxide. More recently Chenouda and El-Awamry (1971a), again using the same solvent system with ascending chromatography, reported Rfs of 0.81-0.84 and 0.57-0.59 for biotin and biotin sulfoxide respectively.

Therefore, it appears that the main component of chromatogram III was biotin-d-sulfoxide with biotin present

in a small amount, whereas the major component of chromatogram IV was biotin, with biotin-d-sulfoxide present as a minor component. Radioactivity, then, is associated with "true" sulfur containing biotin. Because the radioactivity occurs at both the biotin and the biotin-d-sulfoxide spots it seems likely that radioactivity is associated with biotin itself rather that being present in some other component which simply has the same Rf value as biotin.

P. blakesleeanus grown with ^{75}Se-selenite: an attempt to isolate ^{75}Se-labeled selenobiotin, volatilization of selenite

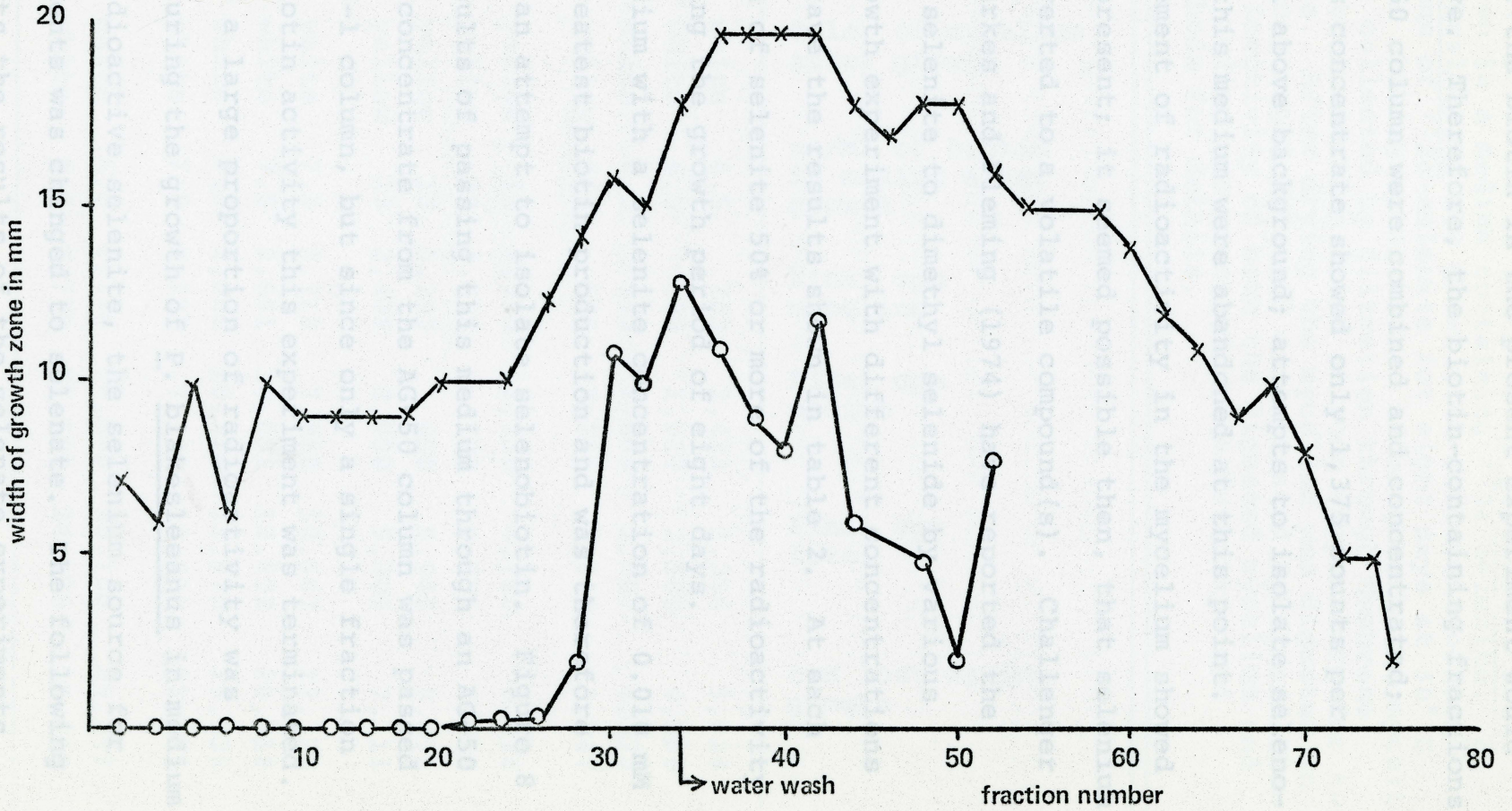
In this experiment an attempt was made to isolate ^{75}Se-labeled selenobiotin from the medium of P. blakesleeanus grown with ^{75}Se-selenite. The procedure for the culture of P. blakesleeanus and the isolation of selenobiotin are given in the Materials and Methods section.

After the medium was passed through an AG-50 column, biotin was eluted as a broad peak (see figure 7); however even though the medium before growth had 2,750,000 counts per minute per ml, none of the fractions from the AG-50 column showed any radioactivity significantly above the background. The absence of detectable levels of radioactivity did not preclude the possibility that selenobiotin was present for two reasons. First there were only 6.8 µg of biotin in 340 ml of medium collected. And secondly even if selenium can substitute for sulfur in biotin only

Figure 7. Separation on an Ag-50 column of biotin active material from P. blakesleeanus medium grown with ^{75}Se-selenite. Five hundred ml of P. blakesleeanus medium with 500 μCi of $H_2{}^{75}SeO_3$ had 2.8×10^5 cpm at the time of inoculation. After growth 340 ml of medium with approximately 20 ng of biotin per ml were collected and passed through an AG-50 column (1 x 16 cm). Ten ml fractions were collected. Fraction 28-58 from this column were combined and concentrated. None of the fractions showed levels of radioactivity significantly higher than the background.

x = yeast plate assay

o = L. plantarum plate assay


20
15
10
5
width of growth zone in mm
10
20
30
40
50
60
70
80
water wash
fraction number

a fraction of the biotin in the present experiment would be radioactive. Therefore, the biotin-containing fractions from the AG-50 column were combined and concentrated; however, this concentrate showed only 1,375 counts per minute per ml above background; attempts to isolate seleno-biotin from this medium were abandoned at this point.

Measurement of radioactivity in the mycelium showed very little present; it seemed possible then, that selenium had been converted to a volatile compound(s). Challenger (1951) and Barkes and Fleming (1974) have reported the reduction of selenite to dimethyl selenide by various fungi. A growth experiment with different concentrations of selenite gave the results shown in table 2. At each concentration of selenite 50% or more of the radioactivity was lost during the growth period of eight days.

The medium with a selenite concentration of 0.010 mM showed the greatest biotin production and was therefore selected for an attempt to isolate selenobiotin. Figure 8 shows the results of passing this medium through an AG-50 column. The concentrate from the AG-50 column was passed through an AG-1 column, but since only a single fraction showed any biotin activity this experiment was terminated.

Because a large proportion of radioactivity was volatilized during the growth of P. blakesleeanus in medium containing radioactive selenite, the selenium source for these experiments was changed to selenate. The following section reports the results of the selenate experiments.

Table 2. Volatilization of selenite by P. blakesleeanus grown in a medium containing ^{75}Se-selenite. P. blakesleeanus was grown with different concentrations of ^{75}Se-selenite for eight days at 30° C in a shaking water bath. After growth the mycelium was collected by filtration through membrane filters and then dried at room temperature for two days. After drying the mycelium was digested in concentrated HNO_3 and concentrated H_2SO_4 and then radioactivity of both the digested mycelium and the medium was measured. See Materials and Methods for details.

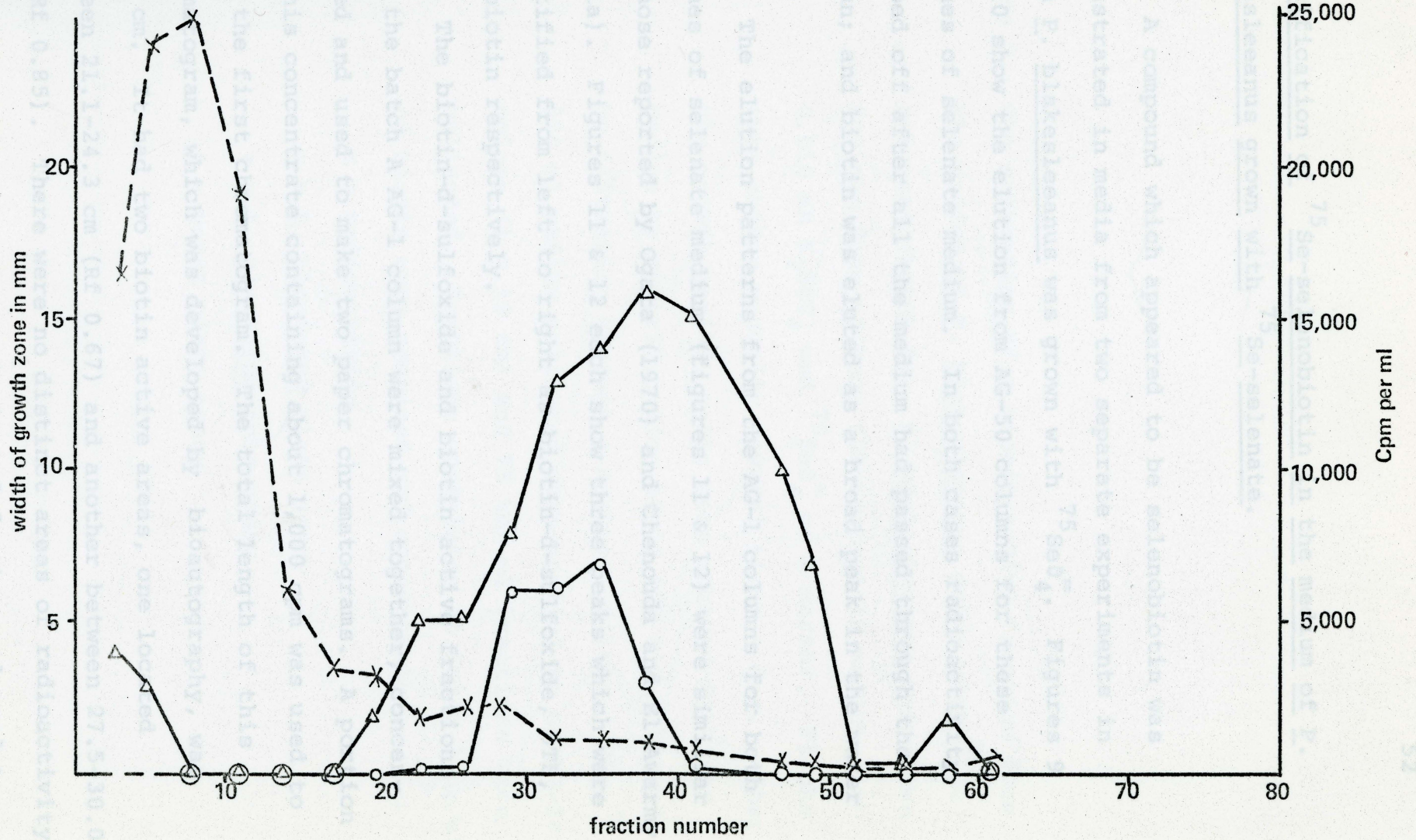
concentration of Na_2SeO_3 in mM	Initial cpm	color of mycelium after growth	ng/ml of biotin in medium after growth	Final cpm mycelium	Final cpm medium	Final cpm total	cpm lost during growth
0.005	7,080	white	6.5	783	2,473	3,256	3,824
0.005	7,080	white	6.5	583	1,917	2,500	4,580
0.005	7,080	white	6.5	843	1,664	2,507	4,573
0.005	7,080	yellow	6.5	555	2,288	2,843	4,247
0.010	13,020	yellow	13	1,980	2,730	4,710	8,310
0.010	13,020	yellow	13	4,878	728	5,606	7,414
0.010	13,020	yellow	13	5,292	650	5,942	7,078
0.010	13,020	yellow	13	2,409	2,900	5,309	7,711
0.020	32,910	white	7.5	2,719	7,868	10,587	22,323
0.020	32,910	white	7.5	2,661	10,052	12,692	20,218
0.020	32,910	white	7.5	2,661	10,314	12,975	19,935
0.020	32,910	white	7.5	3,237	7,398	10,635	22,275
0.040	50,010	white	6.0	6,128	14,580	20,708	29,302
0.040	50,010	white	6.0	2,775	21,600	24,375	25,635

Figure 8. The separation on an AG-50 column of a biotin active fraction from the medium of P. blakesleeanus grown with ^{75}Se-selenite. One hundred twenty ml of P. blakesleeanus medium (containing 0.010 mM Na_2SeO_3 and $^{75}SeO_3^=$ as a marker) from the selenite volatilization experiment was passed through an AG-50 column (1 x 20 cm). Ten ml fractions were collected. Fractions 23-41 were combined and concentrated in preparation for an AG-1 column.

x = radioactivity

o = L. plantarum plate assay

Δ = yeast plate assay


width of growth zone in mm
Cpm per ml
fraction number
5
10
15
20
5,000
10,000
15,000
20,000
25,000
10
20
30
40
50
60
70
80

Identification of ^{75}Se-selenobiotin in the medium of P. blakesleeanus grown with ^{75}Se-selenate.

A compound which appeared to be selenobiotin was demonstrated in media from two separate experiments in which P. blakesleeanus was grown with $^{75}SeO_4^{=}$. Figures 9 and 10 show the elution from AG-50 columns for these batches of selenate medium. In both cases radioactivity dropped off after all the medium had passed through the column; and biotin was eluted as a broad peak in the water wash.

The elution patterns from the AG-1 columns for both batches of selenate medium (figures 11 & 12) were similiar to those reported by Ogata (1970) and Chenouda and El-Awarmy (1971a). Figures 11 & 12 each show three peaks which were identified from left to right as biotin-d-sulfoxide, DTB, and biotin respectively.

The biotin-d-sulfoxide and biotin active fractions from the batch A AG-1 column were mixed together, concentrated and used to make two paper chromatograms. A portion of this concentrate containing about 1,000 cpm was used to make the first chromatogram. The total length of this chromatogram, which was developed by bioautography, was 33.5 cm. It had two biotin active areas, one located between 21.1-24.3 cm (Rf 0.67) and another between 27.5-30.0 cm (Rf 0.85). There were no distinct areas of radioactivity. The remaining sample was concentrated further and used to

Figure 9. The separation of a biotin active fraction from the medium of P. blakesleeanus grown with ^{75}Se-selenate (batch A). P. blakesleeanus was grown for ten days at room temperature in medium with 0.010 mM K_2SeO_4 and $^{75}SeO_4^{=}$ as a marker. Flasks were shaken manually several times a day. After the growth period, cells were removed from the medium by centrifugation at 10,000 x g followed by filtration through a membrane filter. The 350 ml medium collected were applied to an AG-50 column (1 x 19 cm) and 75 ten ml fractions were collected. Fractions containing at least 1 ng/ml of biotin were combined and concentrated.

x = radioactivity

o = L. plantarum plate assay

• = L. plantarum tube assay

Δ = yeast plate assay

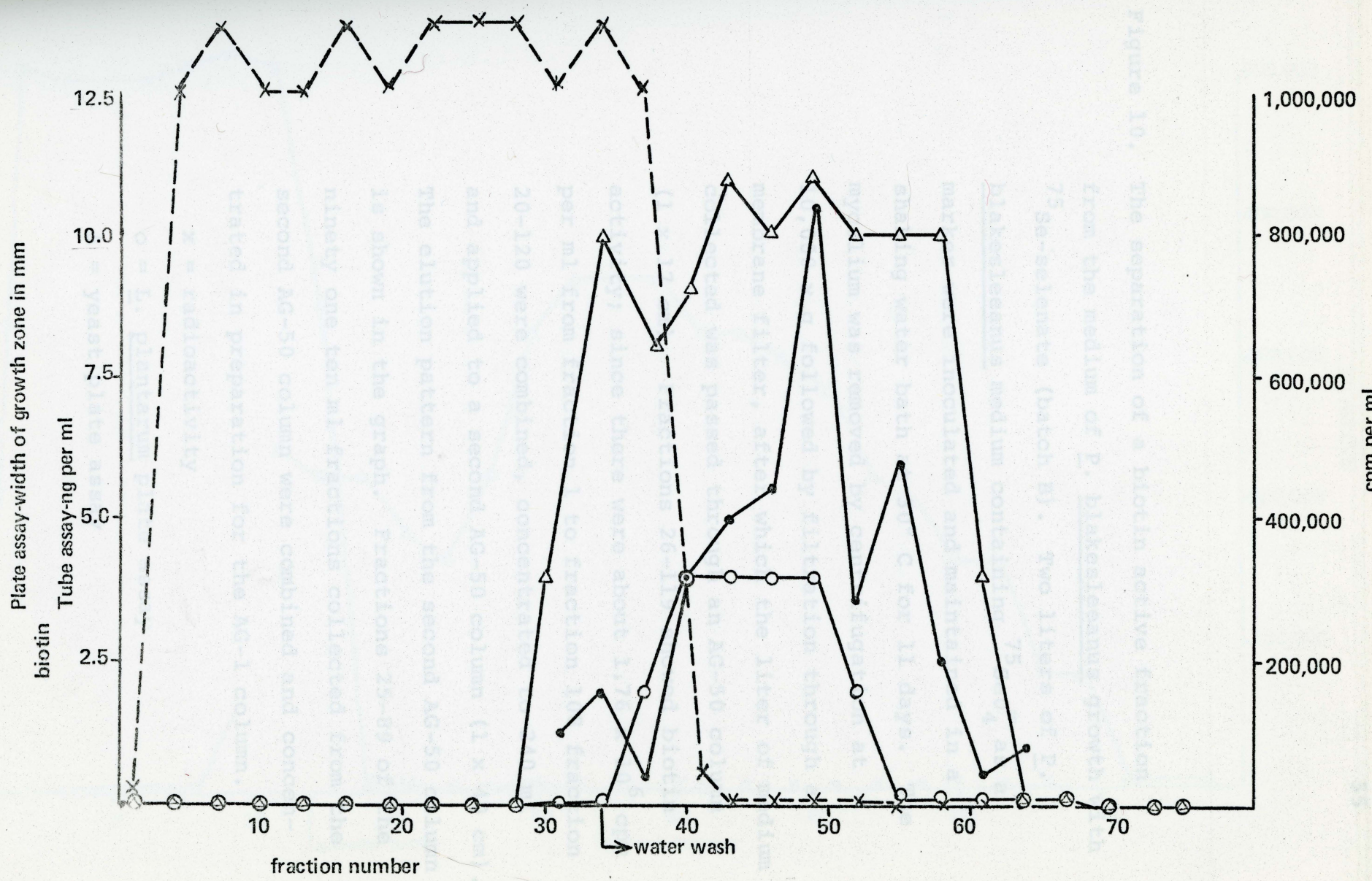


Plate assay-width of growth zone in mm
biotin
Tube assay-ng per ml
12.5
10.0
7.5
5.0
2.5
1,000,000
800,000
600,000
400,000
200,000
cpm per ml
10
20
30
40
50
60
70
water wash
fraction number

Figure 10. The separation of a biotin active fraction from the medium of P. blakesleeanus growth with ^{75}Se-selenate (batch B). Two liters of P. blakesleeanus medium containing $^{75}SeO_4^{=}$ as a marker were inoculated and maintained in a shaking water bath at 30° C for 11 days. The mycelium was removed by centrifugation at 10,000 x g followed by filtration through a membrane filter, after which the liter of medium collected was passed through an AG-50 column (1 x 17 cm). Fractions 26-119 showed biotin activity; since there were about 1.76×10^6 cpm per ml from fraction 1 to fraction 108 fraction 20-120 were combined, concentrated to 240 ml and applied to a second AG-50 column (1 x 40 cm). The elution pattern from the second AG-50 column is shown in the graph. Fractions 25-89 of the ninety one ten ml fractions collected from the second AG-50 column were combined and concentrated in preparation for the AG-1 column.

x = radioactivity

o = L. plantarum plate assay

Δ = yeast plate assay

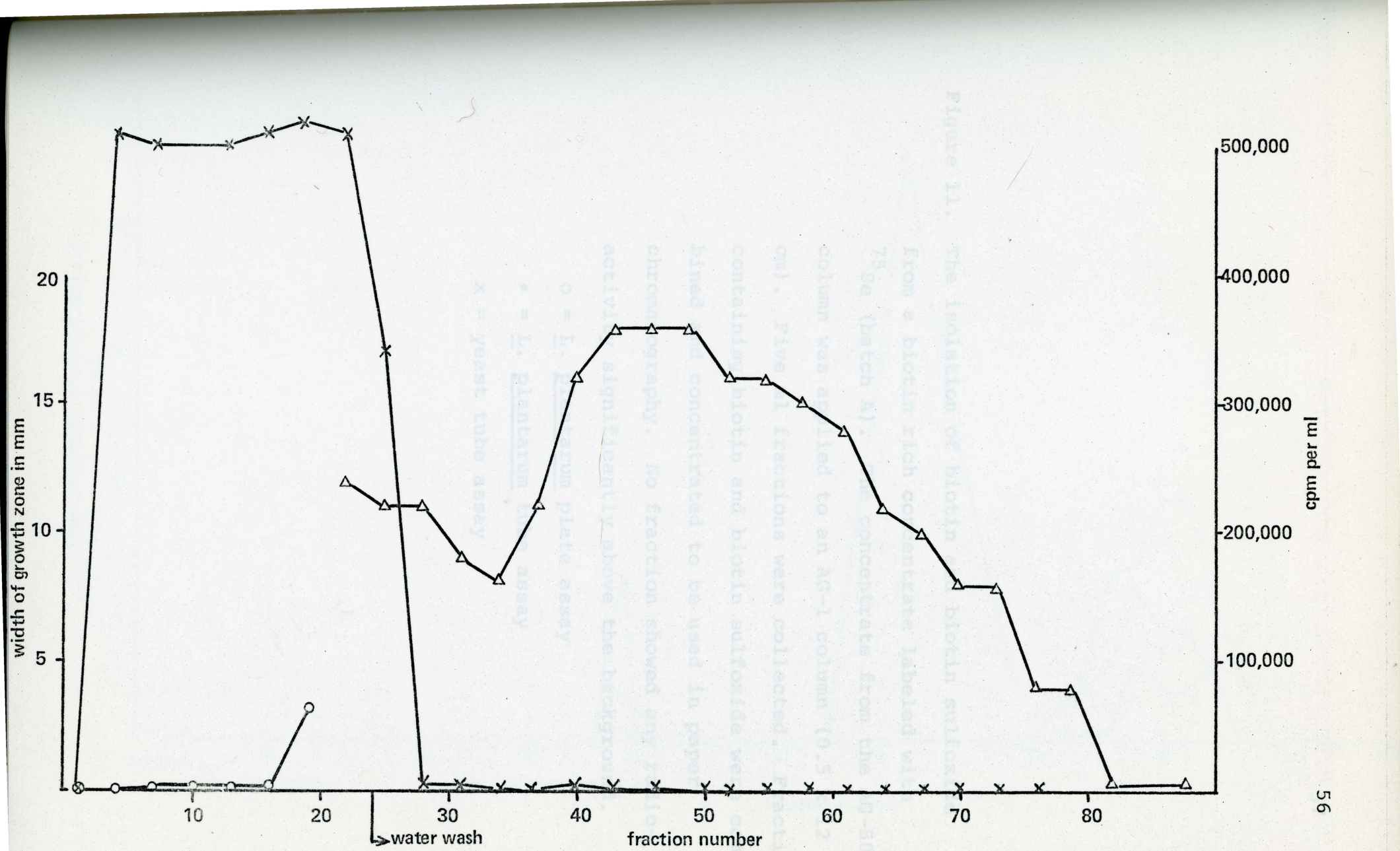

width of growth zone in mm
20
15
10
5
500,000
400,000
300,000
200,000
100,000
cpm per ml
10
20
30
40
50
60
70
80
water wash
fraction number

Figure 11. The isolation of biotin and biotin sulfoxide from a biotin rich concentrate labeled with ^{75}Se (batch A). The concentrate from the AG-50 column was applied to an AG-1 column (0.5 x 12 cm). Five ml fractions were collected. Fraction containing biotin and biotin sulfoxide were combined and concentrated to be used in paper chromatography. No fraction showed any radioactivity significantly above the background.

o = L. plantarum plate assay

• = L. plantarum tube assay

x = yeast tube assay

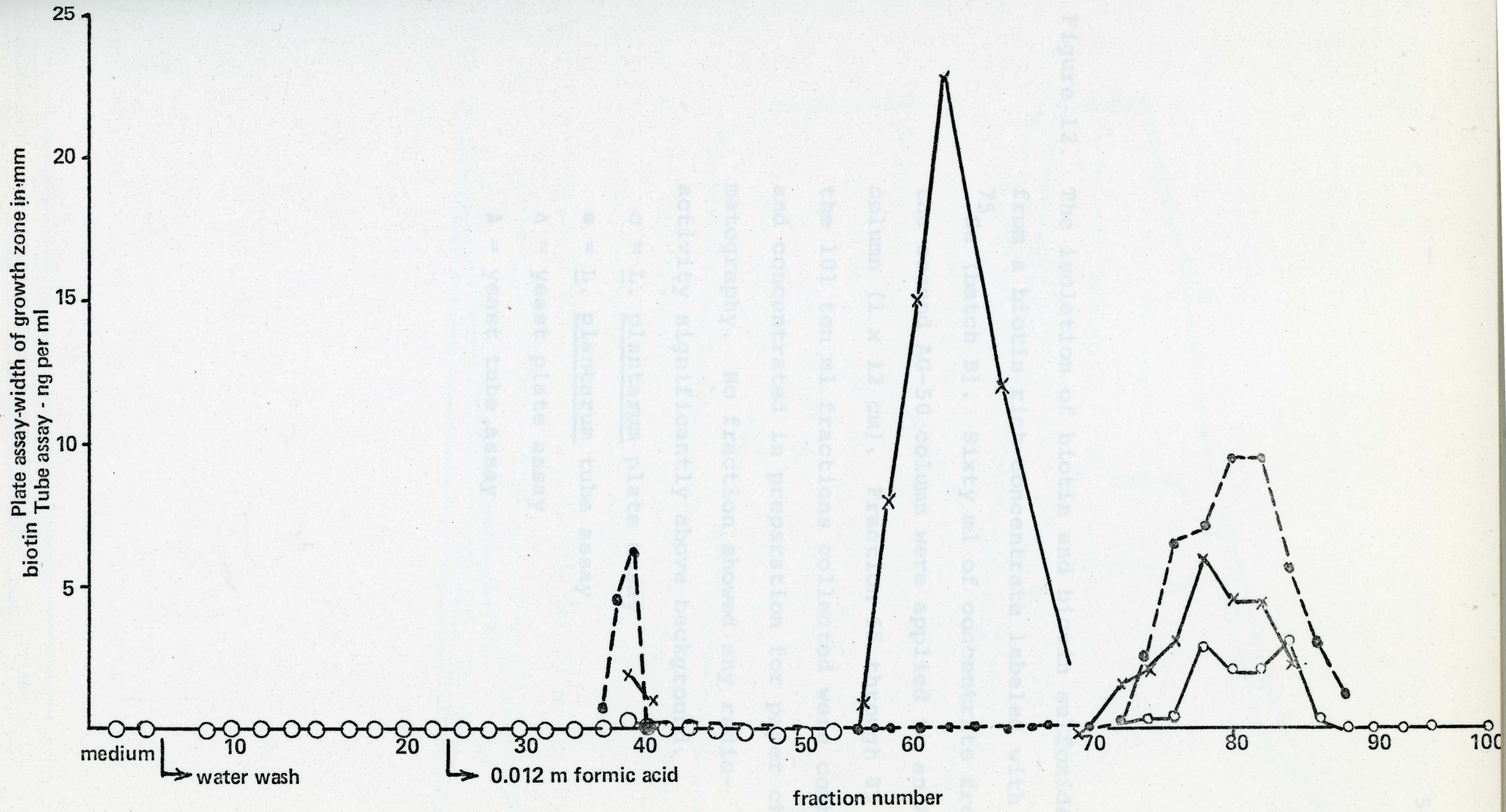

biotin Plate assay-width of growth zone in mm
Tube assay - ng per ml
25
20
15
10
5
medium
water wash
0.012 m formic acid
10
20
30
40
50
60
70
80
90
100
fraction number

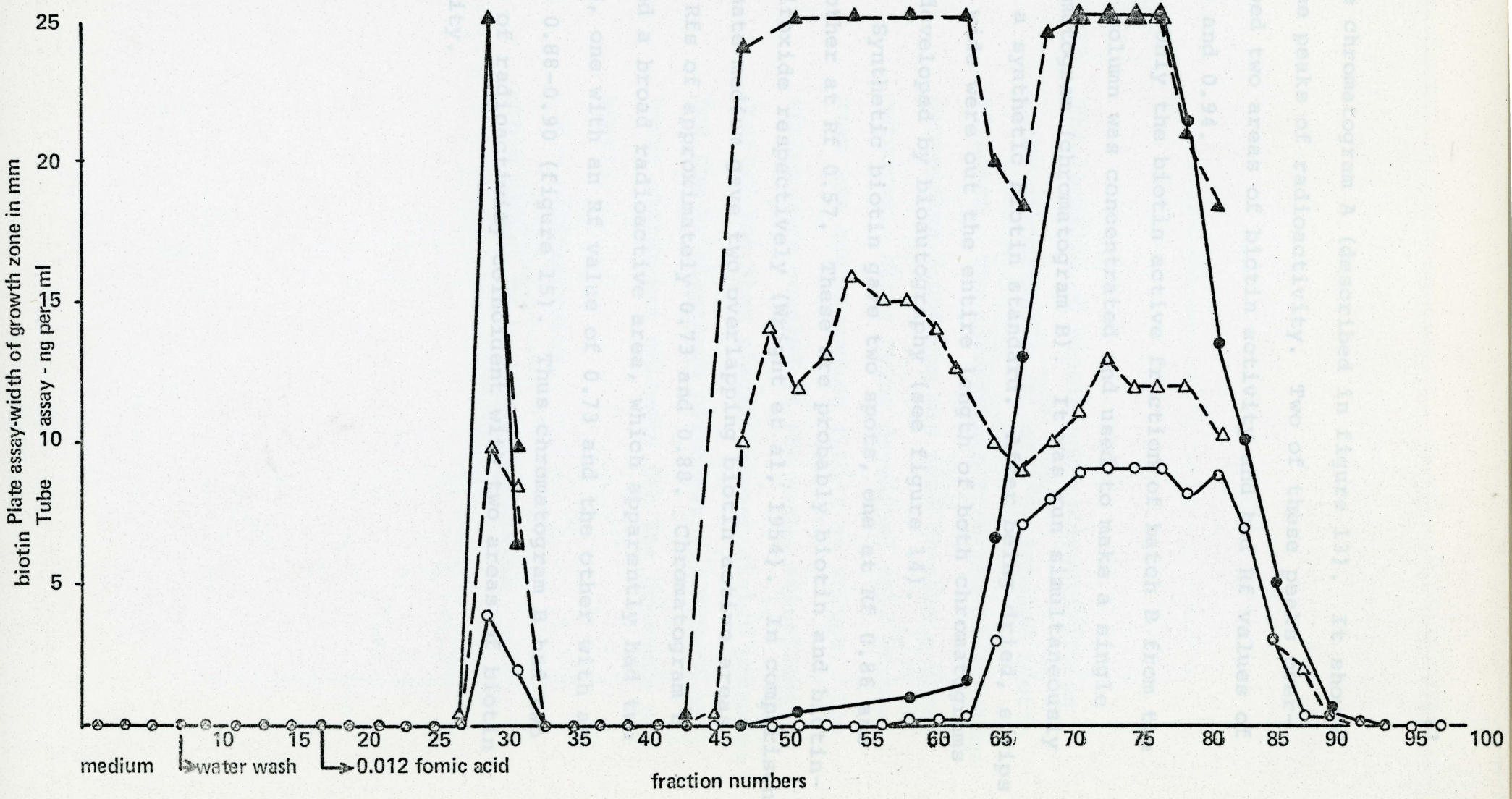
Figure 12. The isolation of biotin and biotin sulfoxide from a biotin rich concentrate labeled with ^{75}Se (batch B). Sixty ml of concentrate from the second AG-50 column were applied to an AG-1 column (1 x 12 cm). Fraction 65 through 85 of the 101 ten ml fractions collected were combined and concentrated in preparation for paper chromatography. No fraction showed any radioactivity significantly above background.

o = L. plantarum plate assay

● = L. plantarum tube assay

Δ = yeast plate assay

▲ = yeast tube assay


biotin Plate assay-width of growth zone in mm
Tube assay - ng per ml
25
20
15
10
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
medium
water wash
0.012 fomic acid
fraction numbers

make chromatogram A (described in figure 13). It showed three peaks of radioactivity. Two of these peaks overlapped two areas of biotin activity and had Rf values of 0.66 and 0.94.

Only the biotin active fractions of batch B from the AG-1 column was concentrated and used to make a single chromatogram (chromatogram B). It was run simultaneously with a synthetic biotin standard. After being dried, strips ½ cm wide were cut the entire length of both chromatograms and developed by bioautography (see figure 14).

Synthetic biotin gave two spots, one at Rf 0.86 and the other at Rf 0.57. These are probably biotin and biotin-d-sulfoxide respectively (Wright et al, 1954). In comparison, selenate medium gave two overlapping biotin active areas with Rfs of approximately 0.73 and 0.88. Chromatogram B showed a broad radioactive area, which apparently had two peaks, one with an Rf value of 0.73 and the other with an Rf of 0.88-0.90 (figure 15). Thus chromatogram B had two areas of radioactivity coincident with two areas of biotin activity.

Figure 13. The location of biotin and radioactivity on a paper chromatogram (A) of ^{75}Se labeled biotin vitamers. The concentrate from the AG-1 column containing 5,000 cpm was used to make a chromatogram. After being developed this chromatogram was cut crosswise into 1 cm strips. The strips were counted in the gamma scintillation counter and then placed on agar plates prepared for the L. plantarum plate assay. Growth on these plates was evidence for the presence of biotin.

x = radioactivity

o = growth on L. plantarum plates

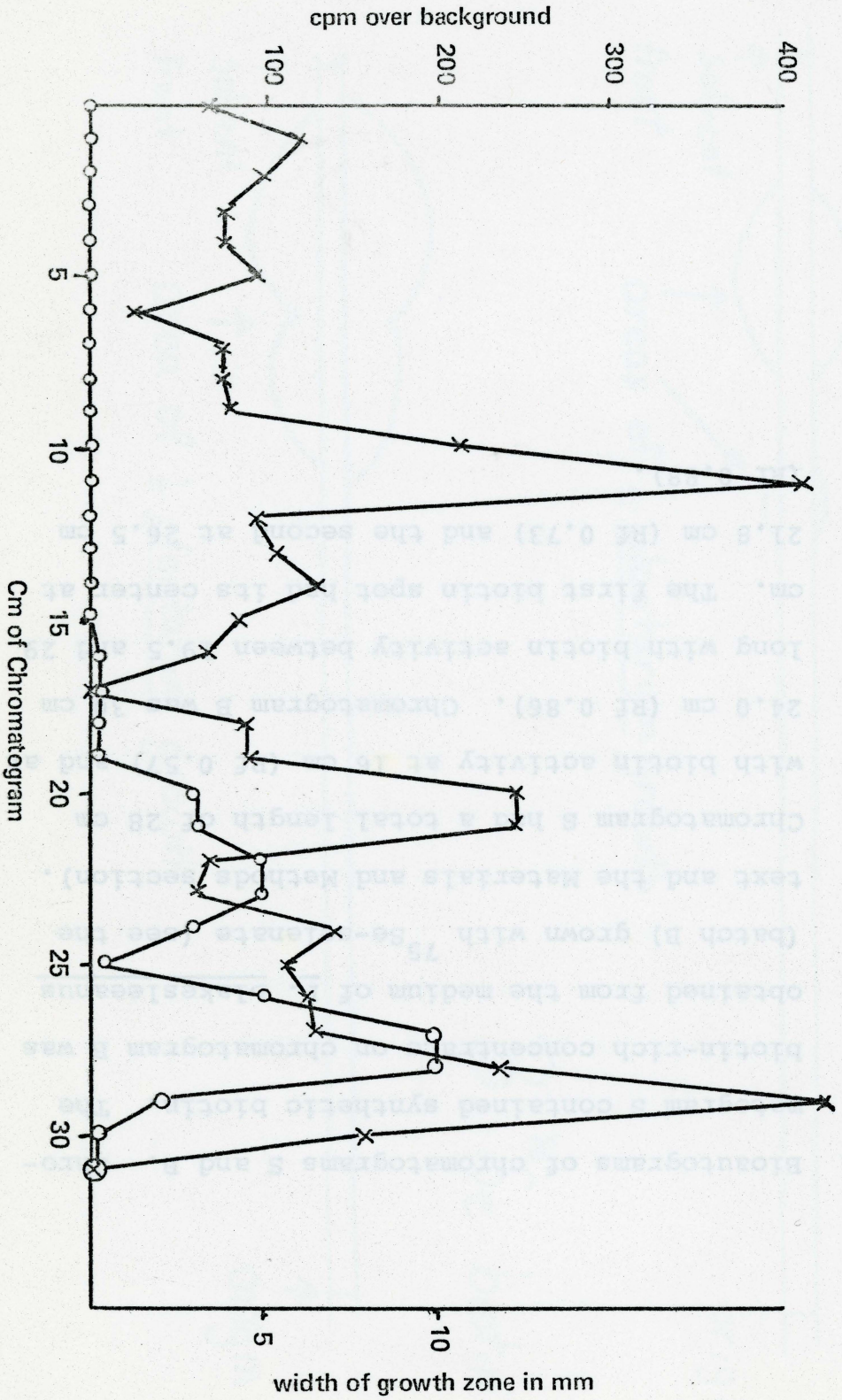
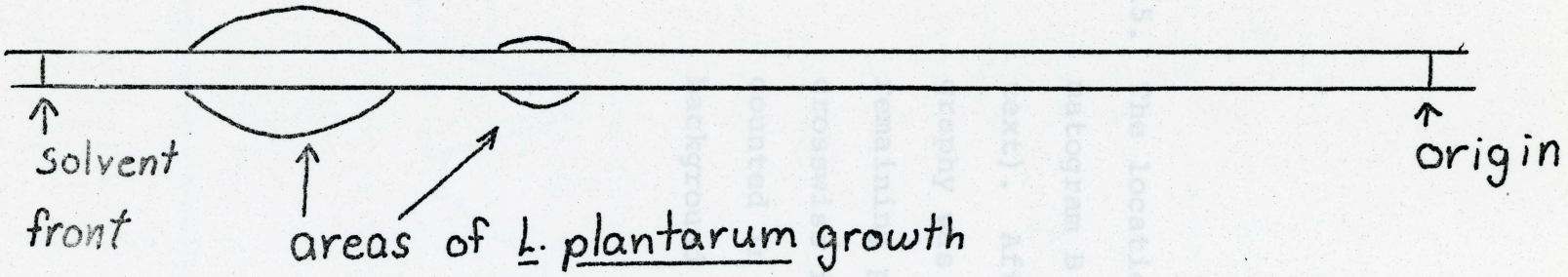
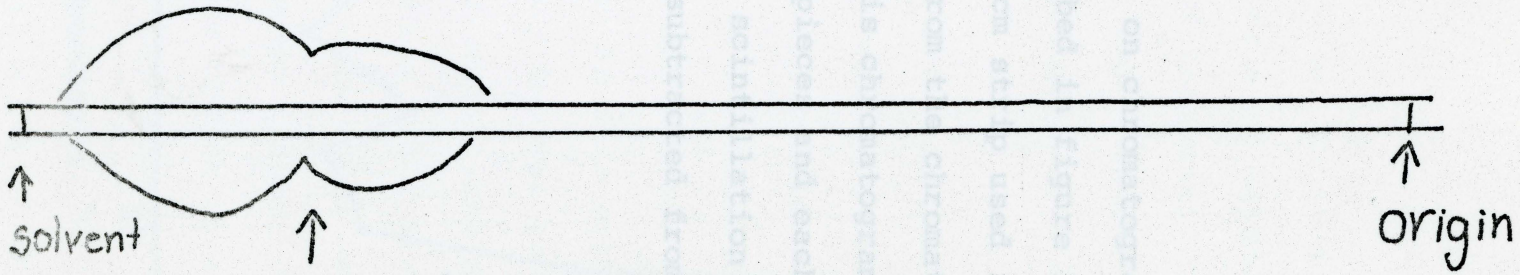

cpm over background
100
200
300
400
5
10
15
20
25
30
Cm of Chromatogram
5
10
width of growth zone in mm

Figure 14. Bioautograms of chromatograms S and B. Chromatogram S contained synthetic biotin. The biotin-rich concentrate on chromatogram B was obtained from the medium of P. blakesleeanus (batch B) grown with ^{75}Se-selenate (see the text and the Materials and Methods section). Chromatogram S had a total length of 28 cm with biotin activity at 16 cm (Rf 0.57) and at 24.0 cm (Rf 0.86). Chromatogram B was 30 cm long with biotin activity between 19.5 and 29 cm. The first biotin spot had its center at 21.8 cm (Rf 0.73) and the second at 26.5 cm (Rf 0.88).

S synthetic biotin



B ^{75}Se biotin



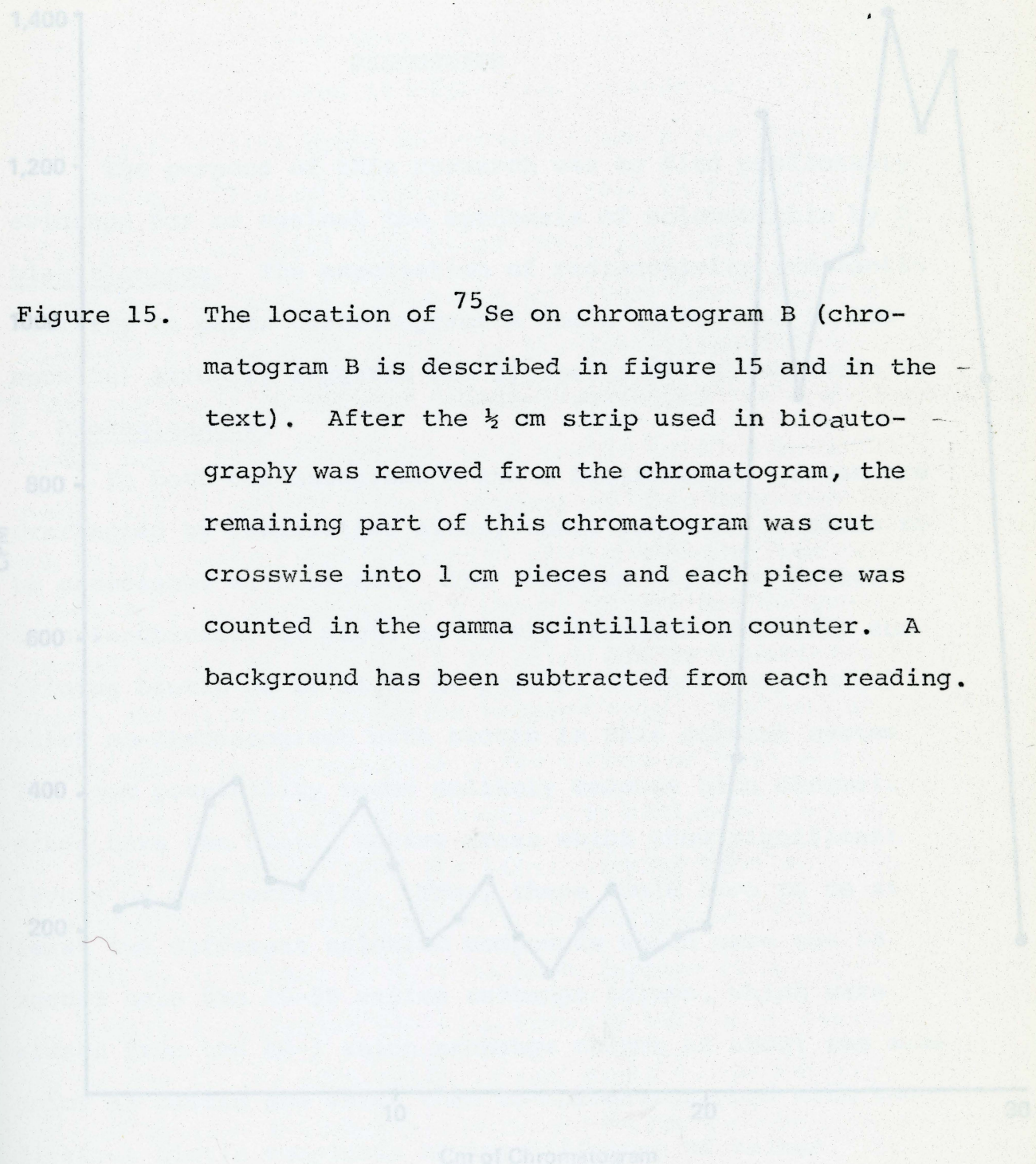
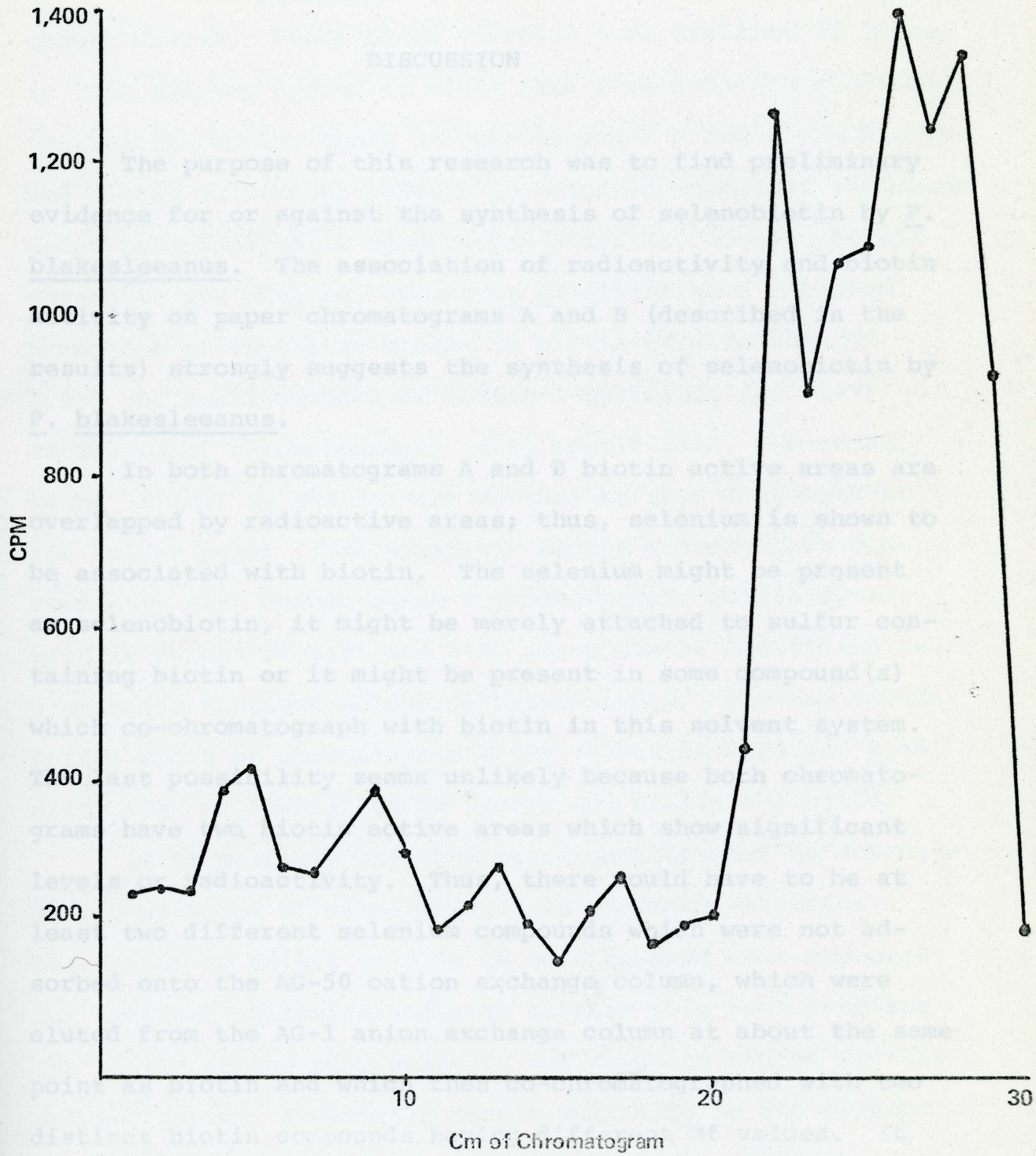


Figure 15. The location of ^{75}Se on chromatogram B (chromatogram B is described in figure 15 and in the text). After the ½ cm strip used in bioautography was removed from the chromatogram, the remaining part of this chromatogram was cut crosswise into 1 cm pieces and each piece was counted in the gamma scintillation counter. A background has been subtracted from each reading.


1,400
1,200
1000
800
600
400
200
CPM
10
20
30
Cm of Chromatogram

DISCUSSION

The purpose of this research was to find preliminary evidence for or against the synthesis of selenobiotin by P. blakesleeanus. The association of radioactivity and biotin activity on paper chromatograms A and B (described in the results) strongly suggests the synthesis of selenobiotin by P. blakesleeanus.

In both chromatograms A and B biotin active areas are overlapped by radioactive areas; thus, selenium is shown to be associated with biotin. The selenium might be present as selenobiotin, it might be merely attached to sulfur containing biotin or it might be present in some compound(s) which co-chromatograph with biotin in this solvent system. The last possibility seems unlikely because both chromatograms have two biotin active areas which show significant levels or radioactivity. Thus, there would have to be at least two different selenium compounds which were not adsorbed onto the AG-50 cation exchange column, which were eluted from the AG-1 anion exchange column at about the same point as biotin and which then co-chromatographed with two distinct biotin compounds having different Rf values. It seems unlikely that compounds meeting all of the above requirements could have been produced.

Biotin and biotin sulfoxide are the only biotin vitamers, active for L. plantarum, which are found in P. blakesleeanus growth medium. Since these vitamers have distinct Rf values in this solvent system it might seem reasonable to conclude that biotin active spots on chromatograms A and B are biotin and biotin sulfoxide. Biotin is oxidized to biotin sulfoxide under relatively mild conditions (Melville, 1954). As discussed in the results, Wright et al (1954) have reported oxidation of synthetic biotin to biotin-d-sulfoxide (Rf 0.55) and small amounts of biotin-l-sulfoxide (Rf 0.47) during paper chromatography (with acetic acid, n-butanol, water (1:4:5) as the solvent system) by some component of the filter paper. The synthetic biotin standard run concurrently with chromatogram B showed biotin active spots with Rf values of 0.86 and 0.57 which can be identified as biotin and biotin-d-sulfoxide respectively. The two biotin active spots on chromatogram A (Rf values of 0.94 and 0.66) might also be identified as biotin and biotin sulfoxide.

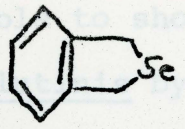
It is unlikely that the biotin active spot on chromatogram B with and Rf value of 0.73 is biotin-d-sulfoxide because an Rf values of 0.73 is much greater than expected for biotin-d-sulfoxide and also because this spot overlaps the biotin active spot which is not known to happen in this solvent system; apparently biotin sulfoxide in not present on chromatogram B.

The absence of biotin-d-sulfoxide might be explained by the following considerations. First, the biotin sulfoxide

fractions from the AG-1 column were not included in concentrate B, but were included in concentrate A. Birnbaum and Pai (1967) have reported the oxidation of biotin to biotin-d-sulfoxide in an uninoculated growth medium prepared for Proteus morganii. These same workers also reported that the presence of sulfhydryl compounds in a complex medium prevents the oxidation of biotin to biotin sulfoxide. Since biotin sulfoxide always appears in the growth medium of P. blakesleeanus (Eisenberg, 1963; Chenouda and El-Awamry, 1971 a), it seems possible that biotin sulfoxide is produced during the growth period and is not produced during paper chromatography due to the presence of some unknown substance. Thus, it may be that the compound with an Rf of 0.73 on chromatogram B is something other than biotin-d-sulfoxide.

Oxybiotin, a synthetic biotin vitamer in which the sulfur atom is replaced by oxygen, has an Rf value of about 0.78 (Wright et al., 1954) with the same solvent system used in these experiments. Oxybiotin is able to stimulate the growth of L. plantarum to about the same extent as biotin. Perhaps the spot on chromatogram B with an Rf value of 0.73 is a selenobiotin.

It is also interesting to speculate about the likelihood that the compound at Rf 0.73 is a biotin selenoxide. There is some evidence that biotin selenoxide might not be as easily formed as biotin sulfoxide and that the selenoxide once formed would prove to be unstable. Saris and Cava (1976) have reported that the oxidation of 2-selenaindane



results in an unstable compound. However the stability of selenobiotin selenoxide can only be speculated about at this time. More concrete information must await the synthesis of selenobiotin selenoxide.

A more precise identification of these selenium containing biotin active compounds isolated from the medium of P. blakesleeanus must await further research. One specific test which might be used to identify selenobiotin is the affinity of biotin for avidin. Avidin, a protein found in egg whites, combines with the ureido structure of biotin or biotin vitamers (Gyorgy, 1954). An experiment using avidin might be carried out in this manner. As in the current experiment, P. blakesleeanus could be grown with (^{75}Se) selenate. After the growth period biotin could be isolated as described in the Materials and Methods Section. Avidin could then be added to the biotin concentrate and allowed to react. Next the biotin-avidin complex could be adsorbed onto bentonite, and the bentonite-avidn-biotin complex could be trapped on a cellulose acetate filter (Wei, 1970). The radioactivity on the filters could then be counted in a gamma scintillation counter. The presence of significant levels of radioactivity would indicate the presence of ^{75}Se-selenobiotin.

Alternatively an experiment might be carried out to determine if selenomethionine is able to stimulate biotin synthesis in P. blakesleeanus. Izumi et al (1973 a) were

able to show stimulation of biotin production in Rhodotorula glutinis by L-methionine in a low sulfate environment. Since selenomethionine is extremely toxic, it would be necessary to determine at what concentration it would significantly impair the growth of P. blakesleeanus. If the concentration of methionine required to stimulate biotin synthesis is below the concentration at which selenomethionine becomes toxic then the following experiment might be undertaken. Growth medium containing suboptimal levels of sulfate would be prepared and divided into three batches. One batch would serve as the control. Methionine would be added to the second batch and selenomethionine to the third. After the growth period biotin concentrations in all three batches would be measured. Levels of biotin in the methionine and selenomethionine batches above those in the control batch would indicate that selenomethionine did stimulate biotin production and would imply that selenobiotin was being formed. Izumi et al (1973 a) have shown the incorporation of ^{35}S from ^{35}S-methionine into biotin in R. glutinis. In addition, if ^{75}Se-selenomethionine were used, an attempt to isolate radioactive selenobiotin from the growth medium could be made. As in the current experiment the coincidence of ^{75}Se and biotin activity would indicate that selenobiotin was present.

SUMMARY

The purpose of these experiments was to explore the possibility that selenium might replace sulfur in biotin synthesized by Phycomyces blakesleeanus. This investigation was carried out by growing P. blakesleeanus in media containing $^{35}SO_4^=$, $^{75}SeO_3^=$, or $^{75}SeO_4^=$. Biotin obtained from the media after growth was isolated and examined for radioactivity. After the growth of P. blakesleeanus in liquid culture, a radioactive biotin-rich concentrate was obtained by passing the media through an AG-50 cation exchange column and then through an AG-1 anion exchange column. The biotin-active fractions from each column were combined and concentrated. The radioactive concentrates were analyzed by paper chromatography. ^{35}S-Labeled compounds were located on the chromatograms with a radiochromatogram scanner, and ^{75}Se-labeled compounds were located by cutting the chromatograms into segments 1 cm wide and counting them in a gamma scintillation counter. Biotin was located by bioautography using L. plantarum.

One paper chromatogram of ^{35}S-labeled biotin concentrate showed both biotin activity and radioactivity at an Rf of 0.88. This spot probably represented ^{35}S-biotin. Another chromatogram showed radioactivity at an Rf of 0.73

and biotin activity at Rf 0.67. These two spots were not coincident, but they did overlap and probably represent ^{35}S-biotin sulfoxide.

When P. blakesleeanus was grown with $^{75}SeO_3^=$ most of the radioactivity disappeared during the growth period. A subsequent experiment with various concentration of ^{75}Se-labeled Na_2SeO_3 showed that about half the radioactivity was volatilized by the end of 8 day's growth.

Efforts to demonstrate ^{75}Se-labeled biotin met with more success when the selenium source was changed from ^{75}Se-selenite to ^{75}Se-selenate. In two separate experiments P. blakesleeanus was grown with ^{75}Se-selenate, after which an attempt was made to demonstrate radioactive biotin in the growth medium as described above. In the first experiment a chromatogram prepared from the biotin concentrate showed two areas of radioactivity with Rf values of 0.66 and 0.94 which overlapped areas of biotin activity. In the second experiment the paper chromatogram showed a broad area of radioactivity with peaks at Rf 0.73 and 0.88-0.90 and two overlapping biotin-active areas with peaks at Rf 0.73 and 0.88. Thus selenium was shown to be associated with biotin synthesized by P. blakesleeanus.

Appendix A

Classification and Life Cycle of *Phycomyces blakesleeanus*

Classification[1] (Ainsworth *et al* 1973)

Kingdom (subkingdom) Eumycota
Class Zygomycetes
Order Mucorales
Family Mucoraceae
Genus Phycomyces
Species blakesleeanus

Life cycle (Alexopoulos, 1962; Stevens, 1974)

Phycomyces blakesleeanus can be cultured in liquid or on agar media. In either case spores give rise to a mass of mycelium from which sporaniophores bearing black sporangia arise during a sexual reproduction.

Sexual reproduction requires the presence of two morphologically indistinguishable mating strains. When the hyphae of opposite strains grow into proximity of each other progametangia develop on each thallus and grow together. Eventually the walls separating the gametangia dissolve and the protoplasts of the two organisms mix forming a zygospore. The zygospore eventually germinates. It is only after

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[1]This classification is not accepted by all authors. Among the more common variations is the inclusion of the Zygomycetes in the larger class Phycomycetes (Bessey, 1950).

germination has begun that karyogany and meiosis take place. Thus the life cycle of P. blakesleeanus is very similar to that of Rhizopus stolenifer the familiar black bread mold which is also a member of the family Mucoraceae.

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